Bioremoval of marker pen inks by exploiting lipase hydrolysis

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Abstract

New and eco-sustainable methods based on the catalytic activity of lipases for the removal of acrylic marker pen inks were investigated. Different biocleaning methodologies were tested using lipases from different sources (viz. bacteria and fungi) dispersed in aqueous systems and microemulsions. Blue, green, red and black marker pens were selected for their chemical composition. Unglazed ceramic substrates were painted using marker pens, and some of these samples were subjected to natural and artificial ageing in order to compare the effectiveness of cleaning methods on fresh and aged ink layers. It was evidenced that acrylic polymer-based inks may be removed with an *oil in water* microemulsion, but cleaning effectiveness was generally enhanced when a lipase was added. Moreover, it was found that all the tests were more effective on the unaged samples, therefore, the cleaning intervention should be performed as soon as possible. Cleaning effectiveness was evaluated by measuring colour differences, acquiring visible reflectance spectra and determining the percentage of white in images of the treated samples by Image J open source software, for the first time used to this purpose.

The results illustrate that only a multi-technique approach can correctly evaluate the effectiveness of different cleaning methods.

Keywords: marker pens, graffiti, biocleaning, lipase, o/w microemulsion

1. Introduction

For about two decades [1,2], biocleaning of stone substrates has been proposed in the field of cultural heritage as an alternative to rather expensive physical treatments (*i.e.*, laser cleaning) and to traditional chemical and mechanical methodologies, which are often toxic and aggressive. Biocleaning methods are usually based on the use of bacteria and enzymes, and have been shown to remove different degradation products from a large variety of substrates, such as stone monuments, wall paintings, and marble statues [1–6], and also to clean up deposits of environmental pollutants [7] and remove synthetic polymers, including those in paints used by graffiti writers [8].

Bacteria have been widely employed in the field of restoration: sulphate crusts have been successfully removed using *Desulfovibrio desulfuricans* [9] and *Desulfovibrio vulgaris* [5,9,10], while *Pseudomonas sutzeri* [11] and *Pseudomonas pseudocaligenes* [10] have been used to eliminate nitrates. Residues of glue used in restoration have been removed using *Pseudomonas sutzeri* [3,12,13], *Desulfovibrio desulfuricans* has also proved able to degrade nitrocellulose-based paints [14] and different kind of *Pseudomonas* (*aeruginosa, stutzeri* and *putida*) have also been tested for the removal of acrylic polymer used in the restoration field [15].

Compared with the cleaning methods based on bacteria, enzymatic techniques appear advantageous because their activity is highly specific, they allow control of pH and time of action, and they leave fewer residues. Enzymes have been used in aqueous formulations, with or without a gel as sorbent, and in ionic liquids (ILs) [16] for the selective removal of protein-, polysaccharide- and lipid-based materials. Literature studies report the use of lipases, amylases, and proteases for cleaning panel and canvas paintings, textiles, and paper artefacts [2,17], but only a few works regard the enzymatic removal of synthetic materials. For instance, a lipolytic enzyme has been used to eliminate aged acrylic resin Paraloid B72. It is thought that cleaning is achieved via hydrolysis of the ester groups of the acrylate and methacrylate units to form free carboxylic acid groups. This chemical modification should lead to an increase in the hydrophilic behaviour of the acrylic resin, facilitating its removal with aqueous cleaning systems [1].

Graffiti materials have a complex chemical composition, including synthetic polymers such as acrylics, alkyds and nitrocellulose and several additives [18–20]. It is generally important to remove non-authorised graffiti as quickly as possible, not only to convey the message that this form of vandalism is unacceptable, but also for a technical reason: the fresher the graffiti, the easier and less damaging its removal. Various methods used to remove graffiti, including traditional chemical and mechanical

approaches and more innovative physical and biological methodologies, have been discussed in two recent reviews [8,21]. It is worth noting that, due to the low solubility of pigments in water, the development of water based, cleaning formulations for stone substrates blotted by graffiti, should focus on the ink binder removal.

This study proposes new enzymatic methods based on the use of lipases to remove graffiti containing acrylic resins from stone substrates. A mechanism similar to that described before for Paraloid B72 (hydrolysis of ester bonds) is expected to help the polymer detachment. Different lipases from fungi and bacteria were tested both in aqueous and in microemulsion systems.

Microemulsions are thermodynamically stable dispersions of two immiscible liquids (*e.g.* water and oil) stabilized by surfactants in form of monophasic transparent fluid. These systems can assure the coexistence of an aqueous phase (in which the lipase is soluble) and an organic phase in which acrylic polymers are soluble. At the same time, suitable microemulsions can ensure lipase activities, but to our knowledge the literature only reports on the stability of lipase entrapped in *water in oil* (w/o) microemulsions (reverse micelles) [22–25]. However, the optimal microemulsion for art-restoration should be *oil in water* (o/w), in which the organic phase required for polymer removal is dispersed as micelles in the aqueous continuous phase where the lipase works. In these conditions, the large interfacial area should facilitate enzyme interaction with the polymeric coating, and the small amount of organic solvent should also reduce the treatment toxicity.

For this reason, we tested two *w/o* and two *o/w* microemulsions with p- xylene as oil and different surfactants. Unglazed white ceramic was chosen as the standard substrate due to its reproducible physical (porosity, roughness surface) and chemical properties from sample to sample; moreover, it can be considered a good mimetic system of stone surfaces, where often graffiti are realized, and its white and uniform colour facilitates the contrast perception, leading to a better evaluation of cleaning effectiveness of the tested procedures.

As to this point several methodologies have been proposed in literature to evaluate cleaning effectiveness [26]. Optical microscopy, scanning electron microscopy (SEM), very often coupled with energy dispersive X-ray (EDX) spectroscopy, Atomic Force Microscopy (AFM), Matrix Assisted Laser Desorption – Time of Flight Mass Spectrometry (MALDI-TOF MS) have been applied in order to determine cleaning selectivity and effectiveness [16,27–30]. Fourier Transform Infrared (FT-IR) spectroscopy has been used to analyse residues after cleaning [29]. Colour differences are also frequently measured in order to study the effects of cleaning treatments [26,30] and in a recent study

hyperspectral imaging has been used as tool to determine the most adequate method of removing graffiti paints applied on granite, variously coloured, rocks [31].

In this study, cleaning effectiveness was evaluated using three different methods in a complementary way: i) acquisition of visible reflectance spectra, ii) measurement of colour differences; iii) determination of the percentage of white in images of the treated areas using IMAGE J open source software. Strengths and weaknesses of the three approaches are also discussed.

2. Experimental

2.1 Materials

Lipases from different sources were used for the cleaning tests which were performed for the removal of acrylic marker pen inks applied on unglazed ceramic substrates.

Lipases

The lipases used for the cleaning tests are the following:

i) a mixture of lipases (MIX) extracted from non-specified bacteria strains (CTS s.r.l., Altavilla Vicentina, Italy; the exact identity of the lipases present in this commercial formulation is unknown);

ii) lipase from *Candida rugosa* (CRL) (L1754 Type VII, ≥700 unit/mg solid);

iii) lipase from *Candida antarctica* (CAL) (≥1.0 U/mg).

Lipases ii) and iii) were purchased from Sigma-Aldrich (Milan, Italy).

Tris base (Trizma® base), Tris Base Hydrochloride (Trizma® hydrochloride)**,** *p*-xylene (purity > 99.0%), Triton X-100, Agar, 1-pentanol (purity ≥ 99%), sodium dodecyl sulphate (SDS) (purity ≥ 98.5%), dioctyl sulphosuccinate sodium salt (AOT) (purity 96%), monobasic and dibasic sodium phosphate, methyl cellulose (average Mn ~40,000, powder, viscosity: 400 cP 274429) and *p*-nitrophenyl butyrate (pNPB) (purity ≥ 98%) were purchased from Sigma-Aldrich (Milan, Italy); Klucel G was obtained from CTS s.r.l. (Altavilla Vicentina, Italy). 2.2.1.1

Spectrophotometric measurements were used to assess the enzymatic activity of the lipase from *Candida rugosa* at various pH values. As regards the *Candida antarctica* lipase, stability conditions (temperature, pH, etc.) have already been investigated by other authors [34], while the mixture of bacterial lipases was used following the manufacturer's instructions on temperature and buffer solution. Lipase activity was evaluated by determining the production rate of p-nitrophenoxide anion via hydrolysis of p-nitrophenylbutyrate (pNPB), at a wavelength of 400 nm (ε = 0,0148 μmol-1cm-1L)

with a JASCO V-530 spectrophotometer. Hydrolysis was conducted at 37°C for 3 minutes and was monitored at different concentrations of pNPB and at various pH values by using buffer solutions.

An aliquot (0.1 mL) of the lipase suspension (0.25 g/L) was added to 0.90 mL of a 0.01 M sodium phosphate buffer solution (pH = 7.2) containing Triton X-100 (0.1 M) and sodium chloride (0.5 M). Solutions were mixed by inversion, placed in the blank and test cuvettes and equilibrated in the spectrophotometer at 37°C. Then 10 µl of substrate solution (50 mM of pNPB in acetonitrile) was added to the test cuvette. A calibration curve was obtained by using standard solutions of 0 (reagent blank), 5, 10, 20, 30 and 40 mM pNPB.

The effect of pH on lipase activity was evaluated using two different buffer solutions (phosphate buffer: pH 5.8 – 7.9; TRIS buffer: pH 8 - 9.5) mixed with Triton X-100 (0.1 M) and sodium chloride (0.5 M). All tests used a 50 mM pNPB solution. Briefly, 0.3 mL of lipase solution (2 mg/ml) was added to 0.90 mL of the reaction buffer before the test. Then the reaction was started by adding 10 µl of pNPB (50 mM).

The CRL hydrolysis of the pNPB follows a Michaelis-Menten behaviour: indeed, the reaction rate increases with the substrate concentration, until saturation for substrate concentration above 50 mM. The activity of CRL at different pH values was found to be maximum at pH 7.9: the plot of the reaction rate, as a function of the pH values, shows a Gaussian distribution, where the maximum represents the optimum pH for the enzyme activity.

Marker pen inks

Blue, green, red and black marker pens of different brands (Uniposca, Tratto and Saratoga) previously characterised by the authors by means of Pyrolysis Gas Chromatography-Mass Spectrometry (Py-GC-MS), FTIR and micro-Raman spectroscopy [19,32] were selected for this work on the basis of their chemical composition (styrene-acrylic resin), as reported in Table 1.

ID	Manufacturer	Colour	Binder	Pigments/Extenders	Additives
P1	UniPosca	Green	Sty-MMA-2EHA-nBA	PG7 - PW6 - PY74	DIOA – PAN
P2	UniPosca	Red	Sty-2EHA-MMA-nBA	PR22 - PW6	PAN
P3	UniPosca	Blue	Sty-MMA-EA	PB15:3	DIBP
P4	Tratto	Red	Sty-EA-MMA	PR11 - PY83	\
P5	Tratto	Green	Sty-nBA	PB15:3 - PY83	\

P6	Saratoga	Black	Sty-αMeSty-MMA	PBk7	\

Legend: αMeSty =-α-methyl styrene; 2EHA = 2-ethylhexyl acrylate; nBA = n-butyl acrylate; DIBP = diisobytil phthalate; DIOA= diisoctyl adipate; EA = ethyl acrylate; MMA = methyl methacrylate; PAN = polyacrylonitrile; Sty = styrene

Table 1. Marker pens used for the biocleaning tests. Binders, pigments/extenders and additives identified by Py-GC-MS (Pyrojector II,SGE; Clarus 680 chromatograph coupled with a Clarus SQ8T single quadrupole mass spectrometer, Perkin Elmer), FTIR spectroscopy (FTS6000 spectrophotometer, BIORAD, equipped with a KBr beamsplitter and DTGS detector) and micro-Raman spectroscopy (Xplora instrument, Horiba JobinYvon, equipped with three lasers (λ_0=532, 638 and 785 nm) and an Olympus BX41 microscope) [19,32].

Test specimens

The unglazed ceramic substrates (10 cm x 10 cm x 0.7 cm) were purchased from the Prodesco de Manises workshop in Valencia (Spain).

The marker pens were used to blot the entire surface of an unglazed ceramic substrate and some samples were subjected to natural and artificial ageing.

Artificial ageing conditions were based on the study of the photo-oxidative effects on similar polymers previously carried out by Favaro *et al*. [33] and according to a comparison between the effects of natural and artificial ageing carried out by one of the authors [32]. Artificial ageing was performed in a QUV BASIC-QPANEL weathering chamber by exposing the test specimens to constant irradiation of 0.35 W/m^2 at a wavelength of λ = 340 nm. Temperature and relative humidity were maintained at constant values of 45°C and 35%, and the ageing cycle was set at 2000 hours.

Natural weathering was performed in Valencia (Spain) for three months (November 2013 - January 2014). In this period the relative moisture varied between 30-85%; whilst the temperature between 2-32°C. The test specimens were positioned facing south and inclined at an angle of 30°.

2.2 Biocleaning methods

The biocleaning tests were carried out on unaged and on naturally and artificially aged test specimens with various lipases in aqueous systems and in oil in water (o/w) microemulsions.

The microemulsions and aqueous systems were applied by brushing 100 μL of the solution onto selected areas (2 cm × 2 cm), and covering the treated area with a 4 mm thick Agar gel. Agar gels at a concentration of 2% (w/v) were prepared by dissolving agar in buffered water at pH 7.9. The solution was heated at 90 °C for 5 minutes, and then the agar dispersion was left to cool until a gel film was obtained. The agar gel was used to ensure that the enzyme remained hydrated. The specimens were

incubated at 38°C for 30 minutes (temperature and time optimized for enzyme activity on the ink substrates). After heating, the dry agar gel was easily detached from the surface. All treated surfaces were washed with distilled water to eliminate residues. For each sample the procedure was repeated twice, since no other significant cleaning effects were observed after additional applications. All biocleaning tests were performed using two aqueous (*AQ1* and *AQ2*) and an *oil in water* microemulsion method (*ME IV*) (see below), with and without the enzymes (Figure 1).

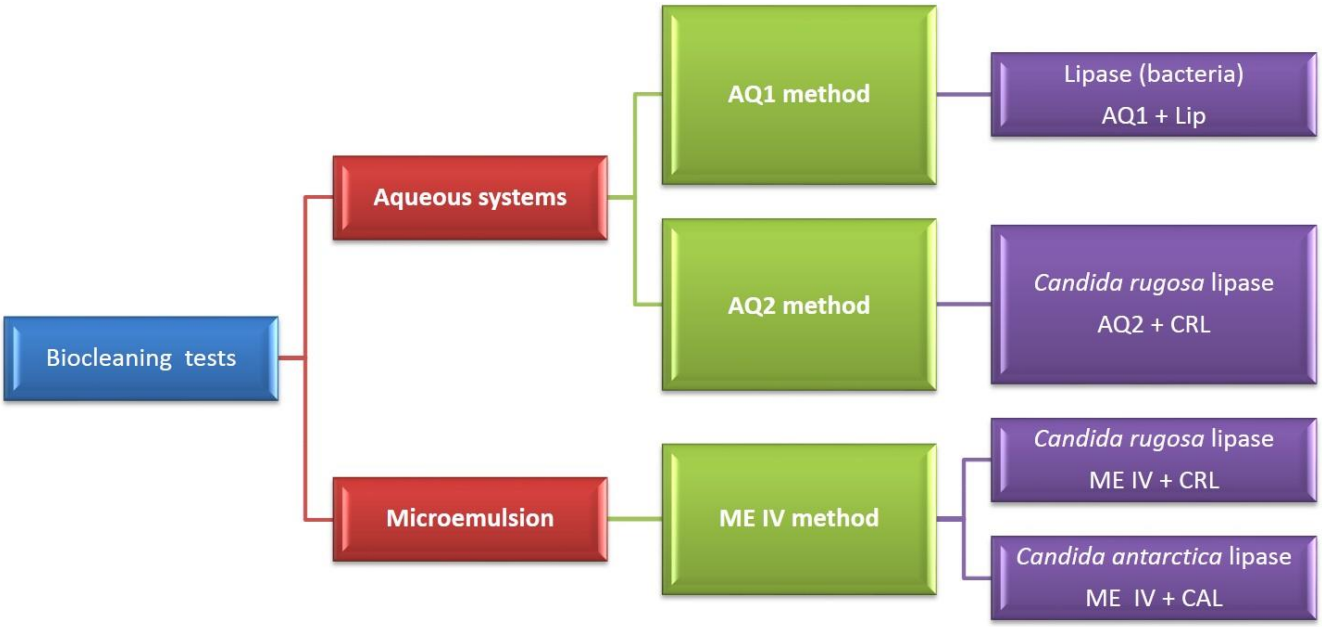

Figure 1. Biocleaning systems

Aqueous systems

The *AQ1* aqueous system was prepared according to CTS srl indications to obtain a mixture of lipases (3% w/v) and Klucel G (hydroxyl propyl cellulose) (2% w/v) in distilled water. *AQ2* was prepared following the indications reported in literature [1]. Briefly, lipase from *Candida rugosa* was dissolved at 0.01% w/v in a Tris-HCl buffer (0.02 M, pH = 7.9, ultrapure water), thickened with methyl cellulose (2% w/v) and kept at 38°C in a water bath. Blank tests were performed using *AQ1* and *AQ2* without the enzyme.

Microemulsions

We tested two *water in oil* (w/o) and two *oil in water* (o/w) microemulsions with *p*-xylene as oil and different surfactants. Surfactants may affect lipase activity by modifying its conformation and/or interfacial properties, thus enhancing enzyme stability. Moreover, surfactants may improve the solubility of the oil phase in water and increase the accessibility of the substrate [34]. In this study the following surfactants were tested: AOT, SDS and Triton X-100. The chemical composition of the microemulsions is reported in Table 2.

Candida rugosa lipase was used for the selection and optimisation of these systems, since it has been shown to retain its functionality with different organic solvents and surfactants [24,25,35]. For the lipolytic activity assay, an aqueous phase composed of a solution of CRL (0.025% w/v) in a TRIS buffer at pH 7.9 (CRL sol.) was used.

ME I	**Composition (w/w %)**	**ME II**	**Composition (w/w %)**	**ME III**	**Composition (w/w %)**	**ME IV**	**Composition (w/w %)**
p-xylene	80.8	**p-xylene**	44.2	**buff. H_2O**	76.7	**buff. H_2O**	86.0
AOT	9.0	**Triton X-100**	29.6	**SDS**	3.7	**Triton X-100**	4.6
CRL sol.	10.2	**1-PeOH**	6.4	**1-PeOH**	7.1	**p-xylene**	0.3
		CRL sol.	19.8	**p-xylene**	2.4	**CRL sol.**	9.1
				CRL sol.	10.1		

Table 2. Composition of *water in oil* (*ME I* and *ME II*) and *oil in water* (*ME III* and *ME IV*) microemulsion systems; 1-PeOH = 1-pentanol

The w/o microemulsions (*ME I* and *II*) were obtained by following the same procedure: the surfactant (AOT or Triton X-100) was first dissolved in the oil phase (*p*-xylene) under stirring, and only in *ME II* the co-surfactant (1-pentanol (1-PeOH)) was added dropwise until a stable system was formed. The CRL solution was added and stirred until a transparent solution was formed.

The o/w microemulsions (*ME III* and *IV*) were selected among those proposed as cleaning media for acrylic coatings by Carretti *et al.* [36,37]. M*E III* was prepared with an SDS surfactant, 1-PeOH co-surfactant and *p*-xylene by following the procedure proposed in literature [37], using CRL solution instead of pure water. *ME IV* was prepared by dissolving Triton X-100 in buffered water (pH 7.9) under stirring to obtain a transparent solution. p-Xylene was then added at room temperature and stirred until a stable system was formed. Ammonium carbonate, as present in the Carretti *et al*. formulation

[36], was discarded because it was not necessary for our purposes. Finally, the CRL solution was added. The activity of the enzyme in microemulsions was ascertained by adding 10 µL of pNPB 50 mM: a positive test was indicated when the solution turned yellow.

The *ME I* contained *p*-xylene as the organic phase and AOT as the surfactant. AOT was selected because it is able to form reverse micelles in many apolar organic solvents containing considerable amounts of water or several other polar solvents, without using a co-surfactant [22,38,39]. Moreover, Otero *et al.* have confirmed the activity of isoenzymes A and B from *Candida rugosa* in a water/AOT/n-heptane system [25]. The present study used *p*-xylene as oil, due to its polarity and ability to dissolve the binders of graffiti materials. The *log P* (P is the partition coefficient) of *p*-xylene is 2.5, which should guarantee sufficient hydrophobicity for CRL activity [35].
However, our tests showed that the CRL in *ME I* was not able to hydrolyse the pNPB substrate*;* the combination of AOT and *p*-xylene probably inhibits the CRL activity. On the contrary, lipase activity was preserved in *ME II*, containing a non-ionic surfactant, such as Triton X-100, 1-PeOH as the co-surfactant, and *p*-xylene, as shown by the yellow colour of the microemulsion when pNPB was added. Our results are in agreement with previous studies where non-ionic surfactants, including Triton X-100, also in mixture with the ionic SDS provided good results [34,40–42].
Despite this positive result obtained with the w/o *ME II*, we wanted to focus on o/w formulations representing eco-sustainable systems with a very low environmental impact. The *ME III* formulation with SDS as the surfactant was selected because it had been successfully used for the solubilisation of acrylic polymers in the field of cultural heritage [36]. However, we found that CRL was not able to hydrolyse the pNPB substrate in this system. The reason of such a loss of functionality is unclear. Denaturation probably occurs because of the large amount of SDS and pentanol; many of the hydrophobic binding sites, which are initially hidden in the tertiary structure, are exposed to the aqueous system [43]. Conversely, the CRL was capable to hydrolyse the test substrate in the o/w *ME IV* formulation, indicating that a *p*-xylene organic phase and Triton X-100 surfactant guarantee the lipase activity. A similar result was obtained with CAL which in *ME IV* maintains a satisfactory level of lipase activity.

We thus decided to perform the microemulsion biocleaning tests by using *ME IV* with both lipases (CRL and CAL). The same microemulsion was also tested without any enzyme.

The impact of the lipase on the structure of *ME IV* was investigated by measuring the radii of the droplet particles with the dynamic light scattering technique (DLS).

DLS measurements were performed using a Zetasizer-NanoS (Malvern) instrument operating with a 4 mW He-Ne laser (λ = 633 nm) and a fixed detector angle of 173° (non-invasive backscattering geometry NIBS™), with the cell holder maintained at 25 °C by a Peltier element. Data were collected after automatic optimisation of the instrumental parameters (attenuator, optics position and number of runs). The autocorrelation function (ACF) of the scattered light intensity was usually the average of 12-16 runs of 10 s each. The ACF of the scattered light intensity was converted into the ACF of the scattered electric field. The latter is the Laplace transform of the intensity weighted size distribution function that was retrieved using a standard regularised non-negative least squares analysis, operated by the software implemented by the manufacturer. The obtained intensity weighted size distribution function represents the fraction of the light intensity scattered by particles of different sizes.

The DLS data showed that the *ME IV* system without lipases is formed by w/o swollen micelles with an average hydrodynamic radius of 14.5±0.5 nm; the size distribution of these micelles is quite broad, with a polydispersity index (PdI) of 0.40±0.01. Addition of lipase to this formulation considerably reduced micelle size: the hydrodynamic radius with the enzyme is 9±1 nm with a very low PdI of 0.18±0.03. Interestingly, the type of lipase does not influence this marked decrease in micelle size; the addition of CRL or CAL actually gives the same micelle size.

This result might be due to the fact that lipase is an interfacial enzyme. The size of an o/w microemulsion micelle depends on the ratio between the amount of solubilized oil and the amount of surfactant at the interphase [44]. Lipase tends to be adsorbed at the water-oil interphase, where it evidently behaves as a surfactant, thus reducing droplet size.

2.3 Evaluation of the biocleaning tests

Stereomicroscope

For each zone subjected to the cleaning treatments, a stereomicroscope LEICA S8APO connected to a Nikon D5700 reflex digital camera took four photographs at different magnifications (1x, 2x, 4x and 8x).

Colour measurement and reflectance spectra

The chromatic properties and the reflectance spectra of the treated and untreated surfaces were measured using an X-Rite-i1-Pro spectrophotometer. Colour shifts were expressed as colourimetric values according to the CIE L*a*b* procedure using the three variables ΔL*ab, Δa* and Δb*. The magnitude of the global colour variation is given by [26]:

$$\Delta E^*_{ab} = \sqrt{(L^*_1 - L^*_2)^2 + (a^*_1 - a^*_2)^2 (b^*_1 - b^*_2)^2}$$

For each zone, the colourimetric data (L*, a*, b*) were an average value of three measurements on five spots. ΔE*ab values are an average of five ΔE*ab values calculated and the standard deviation was estimated on these values. The ΔE*ab were estimated by considering L^*_1, a^*_1 and b^*_1 relative to the untreated area and L^*_2, a^*_2 and b^*_2 of the treated layer. The higher the ΔE*ab, the higher the cleaning effectiveness

Five spots and three measurements were acquired also for the reflectance spectra used to evaluate fading and chromatic variations.

The area of the reflectance spectra (R) was calculated using the integration tool of Origin Lab 8.0 software. For this calculus the mathematical area (the algebraic sum of trapezoids) was selected. The difference between the areas of the reflectance spectra acquired on the treated and untreated zones (ΔR) was used to evaluate the cleaning effectiveness.

Image processing

The photos obtained with the stereomicroscope at 1x magnification were selected as being more representative of the treated area, and were used to calculate the percentage of white as a measure of the cleaned surface. The images were processed using Image J software to calculate the percentage of a selected colour, in this case white, which may be considered indicative of the cleaned areas.

Image processing involved the following procedure: briefly, a 24-bit image was calibrated and then the "thresholding colour" function was applied to equalize the coloured area to a single-level. For example, all green shades were levelled to only one shade. Then the image was converted into an 8-bit image (in greyscale) to apply segmentation by using the "thresholding greyscale" function to distinguish the object from the background. Once the objects were successfully segmented, they could be analysed using the “analyse particles” function to provide the percentage of white areas (%W).

3. Results and discussion

The biocleaning tests were carried out using the lipases in aqueous systems and in the *o/w* microemulsions. In particular, the mixture of bacterial lipases was used in aqueous system *AQ1*, as suggested by the vendor company, whereas CRL was tested in aqueous system *AQ2*. Both CRL and CAL were also tested in the selected o/w microemulsion system (*ME IV*) (Figure 1). All cleaning methods were applied to both aged and non-aged ink films on unglazed ceramic substrates using marker pens (test specimens).

Biocleaning effectiveness was evaluated by 1) the acquisition of reflectance spectra and the calculus of the difference between the spectral areas of the treated (cleaned) and untreated (uncleaned) zones (ΔR); 2) the determination of the colour differences (ΔE*ab) pre- and post-cleaning treatment; 3) image processing to determine the white percentage of the treated (cleaned) areas (%W).

The most relevant results are shown in Table 3. As a general consideration, the data show that the three evaluation methods may be considered quite comparable. In most cases, a high ΔE*ab value corresponds to high values of %W and ΔR. However, all methods show some limitations. Determination of the colour difference (ΔE*ab) provides an overall indication of colour change, which may not be strictly related to the amount of ink removed. Although the ΔE*ab value is frequently used in literature to measure the "degree of cleaning", it should be stressed that the use of this parameter should be limited to the comparison of different cleaning methods on the same substrate/artefact [26]. For this reason, the use of ΔR could help in correcting data interpretation. Indeed, positive values indicate a high reflectance percentage due to the surface of the white area as a result of ink removal. On the contrary, negative ΔR values may be explained by darkening of the area after the cleaning treatment.

The Image J processing that was proposed in this study as an additional evaluation method allowed us to obtain a percentage of white that might also be related to cleaning effectiveness. Unlike the colourimetric measurements this technique allows to estimate cleaning effectiveness by considering an area instead of point measurements. However, calculation of the percentage of white might be influenced by surface roughness, because shadows may disturb the threshold function.

Specific considerations about the effectiveness of the different biocleaning systems for the different inks may be proposed following detailed analysis of the ΔE*ab, ΔR and %W values, and considering the different types of ink (Table 3).

Samples	non-aged				artificially aged				naturally aged			
	%W	ΔE*ab	σ (ΔE*ab)	ΔR	%W	ΔE*ab	σ (ΔE*ab)	ΔR	%W	ΔE*ab	σ (ΔE*ab)	ΔR
P1												
AQ1 + Lip	60	28.0	± 3.2	83	50	24.1	± 1.8	66	35	19.7	1.7	54
AQ1	42	12.4	± 2.2	38	12	5.8	± 0.3	-6	24	5.1	1.1	2
ME IV+ CAL	42	19.8	± 1.3	65	32	25.7	± 1.2	79	14	6.7	1.4	17
ME IV	33	20.7	± 1.8	59	45	27.4	± 2.2	82	23	8.7	0.9	32
ME IV+ CRL	50	27.9	± 1.6	90	57	29.2	± 1.2	94	29	15.0	2.2	44
AQ2 + CRL	22	18.0	± 2.8	68	15	7.8	± 1.1	8	10	4.0	1.3	5
AQ2	16	11.7	± 1.2	18	6	7.3	± 0.9	14	10	10.1	0.7	25
P2												
AQ1 + Lip	21	28.3	± 2.9	46	48	20.17	± 2.3	8	49	8.4	± 1.6	1
AQ1	14	8.1	± 1.9	1	60	8.3	± 0.3	-24	38	4.0	± 1.6	18
ME IV+ CAL	51	27.8	± 0.6	47	49	11.1	± 0.6	-4	42	6.3	± 0.7	10
ME IV	28	22.4	± 2.3	47	41	12.3	± 2.1	-2	53	5.1	± 1.5	1
ME IV+ CRL	46	25.2	± 1.2	40	57	9.1	± 2.0	17	50	5.4	± 1.5	4
AQ2 + CRL	19	11.7	± 0.9	-6	29	13.2	± 2.1	-46	77	3.5	± 1.1	-8
AQ2	11	9.2	± 0.8	-2	30	12.3	± 2.3	-41	50	4.0	± 1.0	3
P3												
AQ1 + Lip	4	4.0	± 1.3	-2	23	5.4	± 0.7	19	77	5.4	± 1.1	18
AQ1	4	4.5	± 0.6	-3	32	7.2	± 0.8	62	28	2.3	± 0.9	7
ME IV+ CAL	88	36.2	± 2.3	101	37	9.7	± 1.6	79	37	6.6	± 1.1	30
ME IV	13	7.2	± 1.2	1	35	10.6	± 1.9	115	32	8.0	± 1.1	42
ME IV + CRL	7	4.3	± 1.2	20	55	18.8	± 2.0	65	72	22.6	± 1.2	75
AQ2 + CRL	5	5.2	± 0.7	2	34	7.3	± 0.8	-2	32	7.6	± 0.8	32
AQ2	10	4.3	± 1.2	-4	28	9.8	± 1.1	91	23	7.0	± 0.8	33
P4												
AQ1 + Lip	41	37.1	± 1.6	51	15	12.0	± 1.3	4	46	22.6	± 1.0	-16
AQ1	5	7.6	± 1.6	-3	19	15.2	± 1.1	-19	21	10.9	± 1.3	-37
ME IV+ CAL	69	35.5	± 0.7	50	22	17.0	± 2.0	14	30	14.3	± 2.6	-38
ME IV	29	28.2	± 1.0	44	29	24.0	± 1.1	-17	30	18.9	± 2.7	-40
ME IV + CRL	35	21.6	± 1.3	40	29	15.3	± 1.2	3	35	12.5	± 2.4	-30
AQ2 + CRL	30	21.2	± 1.6	33	22	19.6	± 1.2	4	28	19.5	± 1.2	-43
AQ2	18	10.6	± 1.2	6	21	18.3	± 1.0	-11	28	19.1	± 1.3	-39
P5												
AQ1 + Lip	9	12.4	±1.1	4	5	8.8	±0.2	2	2	1.3	±0.1	0
AQ1	8	5.8	±1.4	6	5	7.3	±0.4	15	3	1.8	±0.7	2
ME IV+ CAL	13	12.0	± 0.7	-1	1	4.2	±0.4	0.2	5	2.7	±0.0	-2
ME IV	5	6.7	±0.1	4	9	3.2	±0.3	8	2	2.2	±0.4	0
ME IV + CRL	4	6.4	±0.9	-3	6	3.8	±1.3	2	1	1.2	±0.4	-11
AQ2 + CRL	2	4.9	± 0.5	5	1	1.9	±0.4	-3	3	0.7	±0.5	-5

AQ2	2	8.5	± 1.1	8	5	2.9	±0.5	3	4	0.9	±0.5	-9
P6												
AQ1 + Lip	3	6.6	±0.3	-3	7	4.2	±0.2	18	7	7.4	±0.7	4
AQ1	5	6.8	±0.4	-11	4	3.4	±0.0	3	13	6.2	±0.8	15
ME IV+ CAL	4	4.2	±0.8	-11	7	3.4	±0.8	4	11	4.5	±0.2	4
ME IV	2	2.9	±0.1	-2	5	2.3	±0.3	6	3	2.9	±0.0	3
ME IV + CRL	4	4.9	±0.1	-8	4	5.3	±0.4	3	3	3.2	±0.4	3
AQ2 + CRL	0	3.9	±0.2	-14	6	3.8	±0.4	7	4	5.3	±0.5	7
AQ2	1	5.7	±0.3	-15	9	2.4	±0.3	9	1	7.5	±0.2	5

Table 3. %W, ΔE*ab and ΔR values calculated for the different cleaning methods applied to the aged and non-aged marker pen inks.

Uniposca green marker (P1). The cleaning tests performed on sample P1 gave encouraging results, especially on the non-aged samples. The *AQ1* formulation appeared very efficient when the enzyme was added, as shown by the net increase of values in Table 3. In particular, the microemulsions gave good results for the non-aged and artificially aged samples when *Candida rugosa* lipase was used (*ME IV* + CRL).

Results from the artificially aged samples are reported in Figure 2; satisfactory ink removal was achieved using *AQ1* with the enzyme (mix of lipase) and *ME IV* with and without enzymes. It can also be observed that the histograms for the different evaluation methods show the same trend, showing a good agreement with each other. The lower part of the figure reports the reflectance spectra recorded before and after cleaning treatments. It is evident that there was a net increase of values on specimens where cleaning was efficient.

Compared to the artificially aged samples, for the naturally aged ones the cleaning effectiveness decreased for all the tested systems, although the *AQ1* plus enzyme formulation maintained a good level of performance.

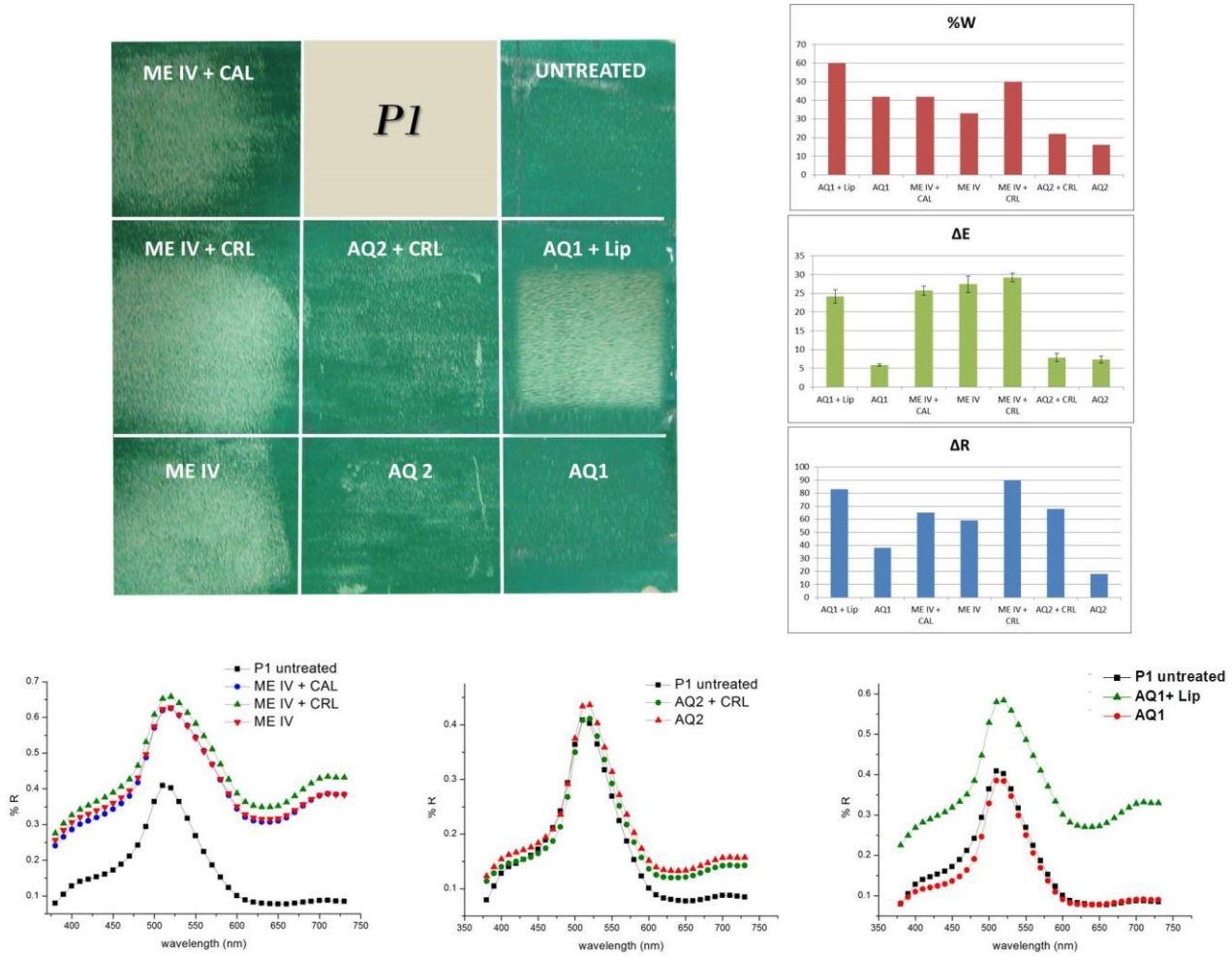

Figure 2. Cleaning tests performed on the artificially aged green Uniposca marker ink (P1). On the left: test specimens with the relevant cleaning system. On the right: histograms of the %W, ΔE*ab and ΔR calculated for each test. Reflectance spectra recorded before and after cleaning treatments with and without enzymes are reported in the lower part of the figure.

Red Uniposca marker (P2). Table 3 shows that the non-aged ink was already successfully removed with the use of bare microemulsions, and that there was a slight enhancement when a lipase was added; *AQ1* was effective only in combination with lipase, and *AQ2* was ineffective with and without the enzyme. The situation changed, however, when the tests involved aged samples. In this case, none of the methods seemed to work.

Results were negative for the aged samples: here it should be pointed out that examination of the reflectance spectra reported in Figure 3 showed that the photo-oxidative ageing already caused a marked loss of red paint. The red marker pen (P2) apparently contains the same type of binder as the

green one (P1) (see Table 1), for which positive cleaning results were obtained on both fresh and aged specimens. However, apart from the type of pigment, Py-GC-MS analysis [19,32] revealed a different relative abundance of the binder monomers: in the green marker ink MMA > 2EHA> nBA, whereas in the red sample 2EHA > MMA > nBA. The difference in sensitivity of acrylates and methacrylates to photo-oxidation could explain the cleaning results for the aged samples. Acrylate units are generally more reactive towards oxidation than methacrylates; moreover, cross-linking reactions prevail over chain scission for long ester side groups [45]. MMA is more abundant in the green marker ink; its photostability is well known and photo-induced oxidation requires severe accelerated ageing conditions [46]. P1 appeared unchanged after artificial ageing in our laboratories, and this facilitated its removal since the ester bonds of the acrylic portion were probably still intact; on the other hand, after natural ageing, the P1 ink layer looked more faded, probably due to the synergistic action of different factors, including relative humidity, temperature, pollution and solar irradiation.

The greater abundance of the less photo-stable 2EHA in the red sample may be responsible for major cross-linking, thus generating a polymer which is more difficult to remove and impeding lipase action.

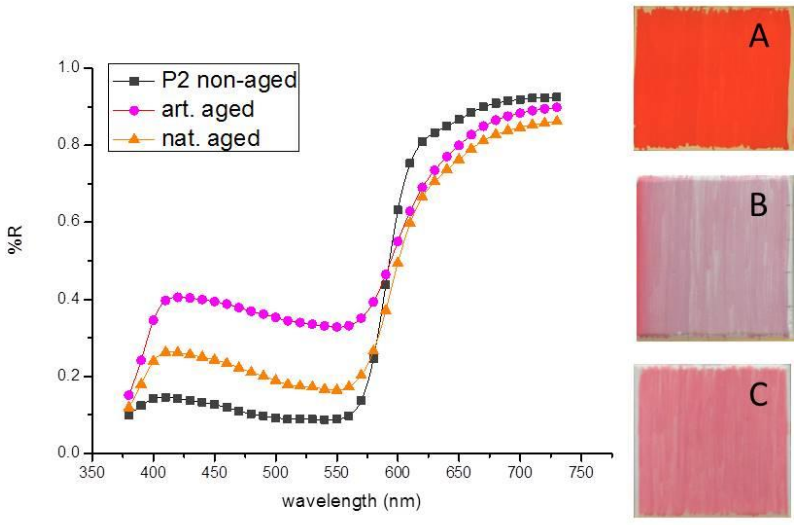

Figure 3. Test specimens with red Uniposca marker pen P2. On the right: before (A) and after artificial (B) and natural (C) ageing. On the left: reflectance spectra recorded on A (squares), B (circles), and C (triangles).

Blue Uniposca marker pen (P3). The data in Table 3 show that for the non-aged samples the addition of CAL in the microemulsion system *ME IV* successfully removes the ink layer, as shown in Figure 4. The CRL and CAL lipases used in this research are known to be non-specific, and their isoenzymes

actually share over 80% of sequence identity; however, they diverge in the lid sequences. In the active enzyme conformation, the open lid participates in substrate-binding and contributes to recognition of the substrate. This might explain the greater activity of CAL compared with CRL on the non-aged P3 sample. On the contrary, CAL activity seems reduced on the aged sample. Concerning the aqueous systems, both *AQ1* and *AQ2* methods, with and without the enzyme, proved ineffective on the aged samples.

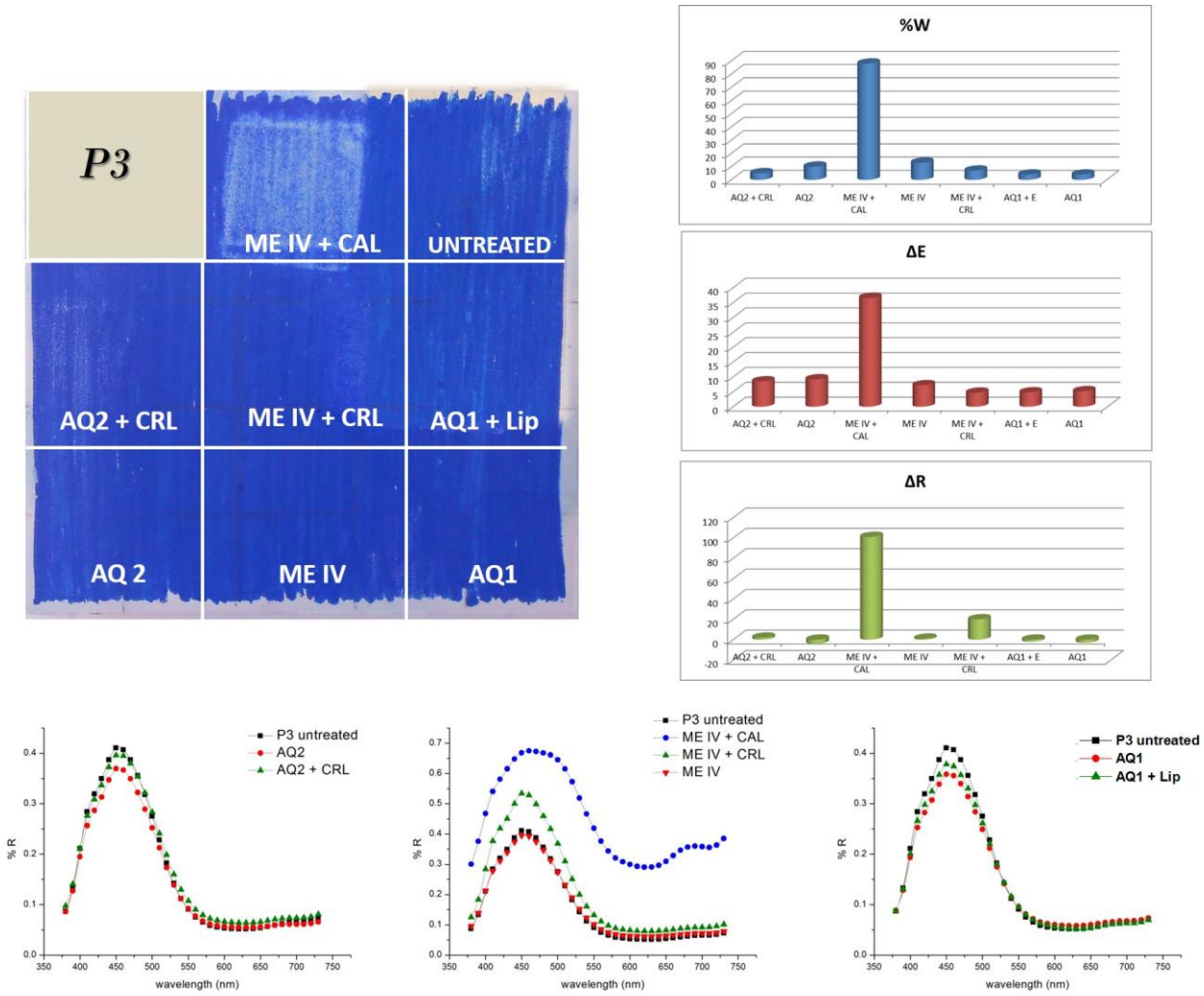

Figure 4. Cleaning tests on the non-aged blue Uniposca marker pen (P3). On the left: Test specimens with the relevant cleaning system. On the right: histograms of the %W, ΔE*ab and ΔR for each test. Reflectance spectra, recorded before and after cleaning treatments with and without enzymes, are reported in the lower part of the figure.

Red Tratto marker pen (P4). From analysis of the data in Table 3 and in Figure 5 regarding the non-aged samples, the aqueous systems with enzymes (*AQ1* + Lip and *AQ2* + CRL) appeared to be the most efficient, and microemulsion *ME IV* + CAL once again performed better than *ME IV* + CRL.

The cleaning effectiveness of *AQ1*+ Lip decreased only for the artificially aged samples, for which the microemulsions and *AQ2* + CRL systems gave good results.

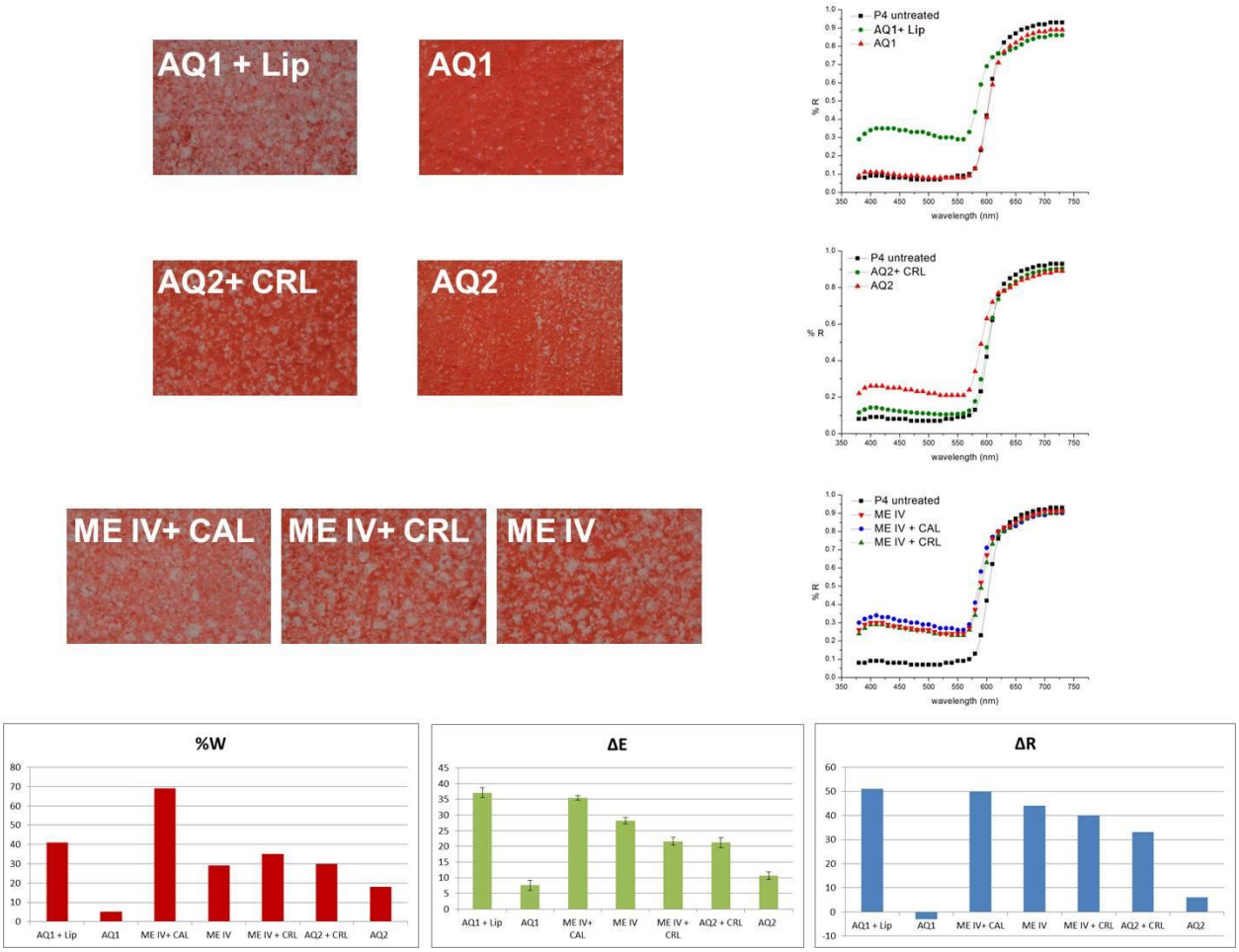

Figure 5. Cleaning tests on the non-aged red Tratto marker pen (P4). On the left: 2X magnification of the areas treated with different cleaning systems. On the right: reflectance spectra recorded before and after biocleaning. Histograms of the %W, ΔE*ab and ΔR for each test are in the lower part of the figure.

It is worthy to note from the results in Table 3 that none of the adopted cleaning methods was successful in removing the **green Tratto marker pen (P5)** or the **black Saratoga marker pen (P6).** In the case of the former, this may be due to the presence of a metallic component; X-ray Photoelectron Spectroscopy identified tin in the ink film (data not shown) and it is well known that this metal can inhibit the enzymatic action.

In the case of P6, the expectedly negative results of the biocleaning tests may be reasonably explained by the low amount of acrylics [19,32] in the binder, which is essentially composed of a copolymer of styrene and α-methyl styrene.

4. Conclusions

The study proposed a sustainable and green approach to the removal of marker pen inks. The method is based on the catalytic activity of the enzyme lipase. Some promising results were obtained in the removal of acrylic polymer-based inks with o/w microemulsions; in few cases these systems were effective even without the enzyme, but cleaning effectiveness was generally enhanced when a lipase was added.

Our findings can be summarized as follows: 1) All tested formulations were more effective on the non-aged samples, suggesting that biocleaning should be performed as soon as possible after blotting. 2) Among the cleaning formulations tested, there is no single formulation that is effective for all kinds of marker pen used, suggesting that simulation specimens should be correctly prepared and tested. 3) Acrylic binders containing a main monomer that is relatively stable to photo-oxidation, such as MMA, are more affordable substrates for lipases than the less photo-stable acrylic resins, possibly because the latter tend to lose the alkyl side chains or are subject to cross-linking which might inhibit the enzymatic attack. This suggests the necessity of a preliminary investigation on the chemical composition of marker pen inks in order to select the most appropriate biocleaning system. 4) The results clearly illustrate that only a multi-technique approach can correctly evaluate the effectiveness of different cleaning methods. To date, there is no standard methodology to evaluate the effectiveness or harmfulness of cleaning procedures, and this makes it difficult to compare results obtained in different conditions (laboratories, cleaning methods, substrates). An attempt to create an unequivocal evaluation method has been made only for laser cleaning methods (UNI 11187-2006), but should be extended to other cleaning techniques. 5) Finally, no cleaning method completely removed the ink from the unglazed ceramic substrates; however optimal results may be achieved by coupling the enzyme based cleaning step with a subsequent very mild mechanical/chemical approach, thus preserving the integrity of the substrate.

In the future, an evaluation of the lipase activity residues with FT-IR spectroscopy will be carried out as well as an estimation of the penetration of the cleaning formulation in the substrate.

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Bioremoval of marker pen inks by exploiting lipase hydrolysis

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Abstract

New and eco-sustainable methods based on the catalytic activity of lipases for the removal of acrylic marker pen inks were investigated. Different biocleaning methodologies were tested using lipases from different sources (viz. bacteria and fungi) dispersed in aqueous systems and microemulsions. Blue, green, red and black marker pens were selected for their chemical composition. Unglazed ceramic substrates were painted using marker pens, and some of these samples were subjected to natural and artificial ageing in order to compare the effectiveness of cleaning methods on fresh and aged ink layers. It was evidenced that acrylic polymer-based inks may be removed with an *oil in water* microemulsion, but cleaning effectiveness was generally enhanced when a lipase was added. Moreover, it was found that all the tests were more effective on the unaged samples, therefore, the cleaning intervention should be performed as soon as possible. Cleaning effectiveness was evaluated by measuring colour differences, acquiring visible reflectance spectra and determining the percentage of white in images of the treated samples by Image J open source software, for the first time used to this purpose.

The results illustrate that only a multi-technique approach can correctly evaluate the effectiveness of different cleaning methods.

Keywords: marker pens, graffiti, biocleaning, lipase, o/w microemulsion

1. Introduction

For about two decades [1,2], biocleaning of stone substrates has been proposed in the field of cultural heritage as an alternative to rather expensive physical treatments (*i.e.*, laser cleaning) and to traditional chemical and mechanical methodologies, which are often toxic and aggressive. Biocleaning methods are usually based on the use of bacteria and enzymes, and have been shown to remove different degradation products from a large variety of substrates, such as stone monuments, wall paintings, and marble statues [1–6], and also to clean up deposits of environmental pollutants [7] and remove synthetic polymers, including those in paints used by graffiti writers [8].

Bacteria have been widely employed in the field of restoration: sulphate crusts have been successfully removed using *Desulfovibrio desulfuricans* [9] and *Desulfovibrio vulgaris* [5,9,10], while *Pseudomonas sutzeri* [11] and *Pseudomonas pseudocaligenes* [10] have been used to eliminate nitrates. Residues of glue used in restoration have been removed using *Pseudomonas sutzeri* [3,12,13], *Desulfovibrio desulfuricans* has also proved able to degrade nitrocellulose-based paints [14] and different kind of *Pseudomonas* (*aeruginosa, stutzeri* and *putida*) have also been tested for the removal of acrylic polymer used in the restoration field [15].

Compared with the cleaning methods based on bacteria, enzymatic techniques appear advantageous because their activity is highly specific, they allow control of pH and time of action, and they leave fewer residues. Enzymes have been used in aqueous formulations, with or without a gel as sorbent, and in ionic liquids (ILs) [16] for the selective removal of protein-, polysaccharide- and lipid-based materials. Literature studies report the use of lipases, amylases, and proteases for cleaning panel and canvas paintings, textiles, and paper artefacts [2,17], but only a few works regard the enzymatic removal of synthetic materials. For instance, a lipolytic enzyme has been used to eliminate aged acrylic resin Paraloid B72. It is thought that cleaning is achieved via hydrolysis of the ester groups of the acrylate and methacrylate units to form free carboxylic acid groups. This chemical modification should lead to an increase in the hydrophilic behaviour of the acrylic resin, facilitating its removal with aqueous cleaning systems [1].

Graffiti materials have a complex chemical composition, including synthetic polymers such as acrylics, alkyds and nitrocellulose and several additives [18–20]. It is generally important to remove non-authorised graffiti as quickly as possible, not only to convey the message that this form of vandalism is

unacceptable, but also for a technical reason: the fresher the graffiti, the easier and less damaging its removal. Various methods used to remove graffiti, including traditional chemical and mechanical approaches and more innovative physical and biological methodologies, have been discussed in two recent reviews [8,21]. It is worth noting that, due to the low solubility of pigments in water, the development of water based, cleaning formulations for stone substrates blotted by graffiti, should focus on the ink binder removal.

This study proposes new enzymatic methods based on the use of lipases to remove graffiti containing acrylic resins from stone substrates. A mechanism similar to that described before for Paraloid B72 (hydrolysis of ester bonds) is expected to help the polymer detachment. Different lipases from fungi and bacteria were tested both in aqueous and in microemulsion systems.

Microemulsions are thermodynamically stable dispersions of two immiscible liquids (*e.g.* water and oil) stabilized by surfactants in form of monophasic transparent fluid. These systems can assure the coexistence of an aqueous phase (in which the lipase is soluble) and an organic phase in which acrylic polymers are soluble. At the same time, suitable microemulsions can ensure lipase activities, but to our knowledge the literature only reports on the stability of lipase entrapped in *water in oil* (w/o) microemulsions (reverse micelles) [22–25]. However, the optimal microemulsion for art-restoration should be *oil in water* (o/w), in which the organic phase required for polymer removal is dispersed as micelles in the aqueous continuous phase where the lipase works. In these conditions, the large interfacial area should facilitate enzyme interaction with the polymeric coating, and the small amount of organic solvent should also reduce the treatment toxicity.

For this reason, we tested two *w/o* and two *o/w* microemulsions with p- xylene as oil and different surfactants. Unglazed white ceramic was chosen as the standard substrate due to its reproducible physical (porosity, roughness surface) and chemical properties from sample to sample; moreover, it can be considered a good mimetic system of stone surfaces, where often graffiti are realized, and its white and uniform colour facilitates the contrast perception, leading to a better evaluation of cleaning effectiveness of the tested procedures.

As to this point several methodologies have been proposed in literature to evaluate cleaning effectiveness [26]. Optical microscopy, scanning electron microscopy (SEM), very often coupled with energy dispersive X-ray (EDX) spectroscopy, Atomic Force Microscopy (AFM), Matrix Assisted Laser Desorption – Time of Flight Mass Spectrometry (MALDI-TOF MS) have been applied in order to determine cleaning selectivity and effectiveness [16,27–30]. Fourier Transform Infrared (FT-IR)

spectroscopy has been used to analyse residues after cleaning [29]. Colour differences are also frequently measured in order to study the effects of cleaning treatments [26,30] and in a recent study hyperspectral imaging has been used as tool to determine the most adequate method of removing graffiti paints applied on granite, variously coloured, rocks [31].

In this study, cleaning effectiveness was evaluated using three different methods in a complementary way: i) acquisition of visible reflectance spectra, ii) measurement of colour differences; iii) determination of the percentage of white in images of the treated areas using IMAGE J open source software. Strengths and weaknesses of the three approaches are also discussed.

2. Experimental

2.1 Materials

Lipases from different sources were used for the cleaning tests which were performed for the removal of acrylic marker pen inks applied on unglazed ceramic substrates.

Lipases

The lipases used for the cleaning tests are the following:

i) a mixture of lipases (MIX) extracted from non-specified bacteria strains (CTS s.r.l., Altavilla Vicentina, Italy; the exact identity of the lipases present in this commercial formulation is unknown);

ii) lipase from *Candida rugosa* (CRL) (L1754 Type VII, ≥700 unit/mg solid);

iii) lipase from *Candida antarctica* (CAL) (≥1.0 U/mg).

Lipases ii) and iii) were purchased from Sigma-Aldrich (Milan, Italy).

Tris base (Trizma® base), Tris Base Hydrochloride (Trizma® hydrochloride), *p*-xylene (purity > 99.0%), Triton X-100, Agar, 1-pentanol (purity ≥ 99%), sodium dodecyl sulphate (SDS) (purity ≥ 98.5%), dioctyl sulphosuccinate sodium salt (AOT) (purity 96%), monobasic and dibasic sodium phosphate, methyl cellulose (average Mn ~40,000, powder, viscosity: 400 cP 274429) and *p*-nitrophenyl butyrate (pNPB) (purity ≥ 98%) were purchased from Sigma-Aldrich (Milan, Italy); Klucel G was obtained from CTS s.r.l. (Altavilla Vicentina, Italy). 2.2.1.1

Spectrophotometric measurements were used to assess the enzymatic activity of the lipase from *Candida rugosa* at various pH values. As regards the *Candida antarctica* lipase, stability conditions (temperature, pH, etc.) have already been investigated by other authors [34], while the mixture of bacterial lipases was used following the manufacturer's instructions on temperature and buffer solution.

Lipase activity was evaluated by determining the production rate of p-nitrophenoxide anion via hydrolysis of p-nitrophenylbutyrate (pNPB), at a wavelength of 400 nm (ε = 0,0148 µmol-1cm-1L) with a JASCO V-530 spectrophotometer. Hydrolysis was conducted at 37°C for 3 minutes and was monitored at different concentrations of pNPB and at various pH values by using buffer solutions.

An aliquot (0.1 mL) of the lipase suspension (0.25 g/L) was added to 0.90 mL of a 0.01 M sodium phosphate buffer solution (pH = 7.2) containing Triton X-100 (0.1 M) and sodium chloride (0.5 M). Solutions were mixed by inversion, placed in the blank and test cuvettes and equilibrated in the spectrophotometer at 37°C. Then 10 µl of substrate solution (50 mM of pNPB in acetonitrile) was added to the test cuvette. A calibration curve was obtained by using standard solutions of 0 (reagent blank), 5, 10, 20, 30 and 40 mM pNPB.

The effect of pH on lipase activity was evaluated using two different buffer solutions (phosphate buffer: pH 5.8 – 7.9; TRIS buffer: pH 8 - 9.5) mixed with Triton X-100 (0.1 M) and sodium chloride (0.5 M). All tests used a 50 mM pNPB solution. Briefly, 0.3 mL of lipase solution (2 mg/ml) was added to 0.90 mL of the reaction buffer before the test. Then the reaction was started by adding 10 µl of pNPB (50 mM).

The CRL hydrolysis of the pNPB follows a Michaelis-Menten behaviour: indeed, the reaction rate increases with the substrate concentration, until saturation for substrate concentration above 50 mM. The activity of CRL at different pH values was found to be maximum at pH 7.9: the plot of the reaction rate, as a function of the pH values, shows a Gaussian distribution, where the maximum represents the optimum pH for the enzyme activity.

Marker pen inks

Blue, green, red and black marker pens of different brands (Uniposca, Tratto and Saratoga) previously characterised by the authors by means of Pyrolysis Gas Chromatography-Mass Spectrometry (Py-GC-MS), FTIR and micro-Raman spectroscopy [19,32] were selected for this work on the basis of their chemical composition (styrene-acrylic resin), as reported in Table 1.

ID	Manufacturer	Colour	Binder	Pigments/Extenders	Additives
P1	UniPosca	Green	Sty-MMA-2EHA-nBA	PG7 - PW6 - PY74	DIOA – PAN
P2	UniPosca	Red	Sty-2EHA-MMA-nBA	PR22 - PW6	PAN
P3	UniPosca	Blue	Sty-MMA-EA	PB15:3	DIBP

P4	Tratto	Red	Sty-EA-MMA	PR11 - PY83	\
P5	Tratto	Green	Sty-nBA	PB15:3 - PY83	\
P6	Saratoga	Black	Sty-αMeSty-MMA	PBk7	\

Legend: αMeSty =-α-methyl styrene; 2EHA = 2-ethylhexyl acrylate; nBA = n-butyl acrylate; DIBP = diisobytil phthalate; DIOA= diisoctyl adipate; EA = ethyl acrylate; MMA = methyl methacrylate; PAN = polyacrylonitrile; Sty = styrene

Table 1. Marker pens used for the biocleaning tests. Binders, pigments/extenders and additives identified by Py-GC-MS (Pyrojector II,SGE; Clarus 680 chromatograph coupled with a Clarus SQ8T single quadrupole mass spectrometer, Perkin Elmer), FTIR spectroscopy (FTS6000 spectrophotometer, BIORAD, equipped with a KBr beamsplitter and DTGS detector) and micro-Raman spectroscopy (Xplora instrument, Horiba JobinYvon, equipped with three lasers (λ_0=532, 638 and 785 nm) and an Olympus BX41 microscope) [19,32].

Test specimens

The unglazed ceramic substrates (10 cm x 10 cm x 0.7 cm) were purchased from the Prodesco de Manises workshop in Valencia (Spain).

The marker pens were used to blot the entire surface of an unglazed ceramic substrate and some samples were subjected to natural and artificial ageing.

Artificial ageing conditions were based on the study of the photo-oxidative effects on similar polymers previously carried out by Favaro *et al*. [33] and according to a comparison between the effects of natural and artificial ageing carried out by one of the authors [32]. Artificial ageing was performed in a QUV BASIC-QPANEL weathering chamber by exposing the test specimens to constant irradiation of 0.35 W/m^2 at a wavelength of λ = 340 nm. Temperature and relative humidity were maintained at constant values of 45°C and 35%, and the ageing cycle was set at 2000 hours.

Natural weathering was performed in Valencia (Spain) for three months (November 2013 - January 2014). In this period the relative moisture varied between 30-85%; whilst the temperature between 2-32°C. The test specimens were positioned facing south and inclined at an angle of 30°.

2.3 Biocleaning methods

The biocleaning tests were carried out on unaged and on naturally and artificially aged test specimens with various lipases in aqueous systems and in oil in water (o/w) microemulsions.

The microemulsions and aqueous systems were applied by brushing 100 µL of the solution onto selected areas (2 cm × 2 cm), and covering the treated area with a 4 mm thick Agar gel. Agar gels at a concentration of 2% (w/v) were prepared by dissolving agar in buffered water at pH 7.9. The solution

was heated at 90 °C for 5 minutes, and then the agar dispersion was left to cool until a gel film was obtained. The agar gel was used to ensure that the enzyme remained hydrated. The specimens were incubated at 38°C for 30 minutes (temperature and time optimized for enzyme activity on the ink substrates). After heating, the dry agar gel was easily detached from the surface. All treated surfaces were washed with distilled water to eliminate residues. For each sample the procedure was repeated twice, since no other significant cleaning effects were observed after additional applications. All biocleaning tests were performed using two aqueous (*AQ1* and *AQ2*) and an *oil in water* microemulsion method (*ME IV*) (see below), with and without the enzymes (Figure 1).

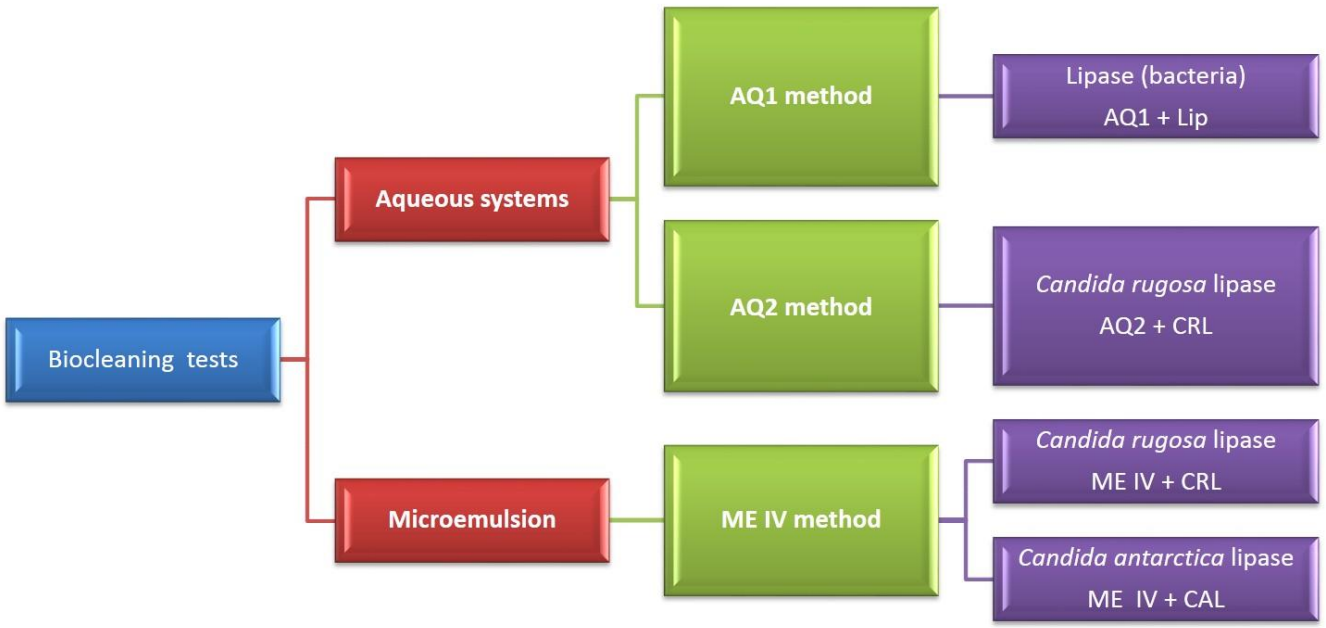

Figure 1. Biocleaning systems

Aqueous systems

The *AQ1* aqueous system was prepared according to CTS srl indications to obtain a mixture of lipases (3% w/v) and Klucel G (hydroxyl propyl cellulose) (2% w/v) in distilled water. *AQ2* was prepared following the indications reported in literature [1]. Briefly, lipase from *Candida rugosa* was dissolved at 0.01% w/v in a Tris-HCl buffer (0.02 M, pH = 7.9, ultrapure water), thickened with methyl cellulose (2% w/v) and kept at 38°C in a water bath. Blank tests were performed using *AQ1* and *AQ2* without the enzyme.

Microemulsions

We tested two *water in oil* (w/o) and two *oil in water* (o/w) microemulsions with *p*-xylene as oil and different surfactants. Surfactants may affect lipase activity by modifying its conformation and/or interfacial properties, thus enhancing enzyme stability. Moreover, surfactants may improve the solubility of the oil phase in water and increase the accessibility of the substrate [34]. In this study the following surfactants were tested: AOT, SDS and Triton X-100. The chemical composition of the microemulsions is reported in Table 2.

Candida rugosa lipase was used for the selection and optimisation of these systems, since it has been shown to retain its functionality with different organic solvents and surfactants [24,25,35]. For the lipolytic activity assay, an aqueous phase composed of a solution of CRL (0.025% w/v) in a TRIS buffer at pH 7.9 (CRL sol.) was used.

ME I	Composition (w/w %)	ME II	Composition (w/w %)	ME III	Composition (w/w %)	ME IV	Composition (w/w %)
p-xylene	80.8	**p-xylene**	44.2	**buff. H_2O**	76.7	**buff. H_2O**	86.0
AOT	9.0	**Triton X-100**	29.6	**SDS**	3.7	**Triton X-100**	4.6
CRL sol.	10.2	**1-PeOH**	6.4	**1-PeOH**	7.1	**p-xylene**	0.3
		CRL sol.	19.8	**p-xylene**	2.4	**CRL sol.**	9.1
				CRL sol.	10.1		

Table 2. Composition of *water in oil* (*ME I* and *ME II*) and *oil in water* (*ME III* and *ME IV*) microemulsion systems; 1-PeOH = 1-pentanol

The w/o microemulsions (*ME I* and *II*) were obtained by following the same procedure: the surfactant (AOT or Triton X-100) was first dissolved in the oil phase (*p*-xylene) under stirring, and only in *ME II* the co-surfactant (1-pentanol (1-PeOH)) was added dropwise until a stable system was formed. The CRL solution was added and stirred until a transparent solution was formed.

The o/w microemulsions (*ME III* and *IV*) were selected among those proposed as cleaning media for acrylic coatings by Carretti *et al.* [36,37]. M*E III* was prepared with an SDS surfactant, 1-PeOH co-surfactant and *p*-xylene by following the procedure proposed in literature [37], using CRL solution instead of pure water. *ME IV* was prepared by dissolving Triton X-100 in buffered water (pH 7.9) under stirring to obtain a transparent solution. p-Xylene was then added at room temperature and stirred until a stable system was formed. Ammonium carbonate, as present in the Carretti *et al*. formulation

[36], was discarded because it was not necessary for our purposes. Finally, the CRL solution was added. The activity of the enzyme in microemulsions was ascertained by adding 10 µL of pNPB 50 mM: a positive test was indicated when the solution turned yellow.

The *ME I* contained *p*-xylene as the organic phase and AOT as the surfactant. AOT was selected because it is able to form reverse micelles in many apolar organic solvents containing considerable amounts of water or several other polar solvents, without using a co-surfactant [22,38,39]. Moreover, Otero *et al.* have confirmed the activity of isoenzymes A and B from *Candida rugosa* in a water/AOT/n-heptane system [25]. The present study used *p*-xylene as oil, due to its polarity and ability to dissolve the binders of graffiti materials. The *log P* (P is the partition coefficient) of *p*-xylene is 2.5, which should guarantee sufficient hydrophobicity for CRL activity [35].
However, our tests showed that the CRL in *ME I* was not able to hydrolyse the pNPB substrate*;* the combination of AOT and *p*-xylene probably inhibits the CRL activity. On the contrary, lipase activity was preserved in *ME II*, containing a non-ionic surfactant, such as Triton X-100, 1-PeOH as the co-surfactant, and *p*-xylene, as shown by the yellow colour of the microemulsion when pNPB was added. Our results are in agreement with previous studies where non-ionic surfactants, including Triton X-100, also in mixture with the ionic SDS provided good results [34,40–42].
Despite this positive result obtained with the w/o *ME II*, we wanted to focus on o/w formulations representing eco-sustainable systems with a very low environmental impact. The *ME III* formulation with SDS as the surfactant was selected because it had been successfully used for the solubilisation of acrylic polymers in the field of cultural heritage [36]. However, we found that CRL was not able to hydrolyse the pNPB substrate in this system. The reason of such a loss of functionality is unclear. Denaturation probably occurs because of the large amount of SDS and pentanol; many of the hydrophobic binding sites, which are initially hidden in the tertiary structure, are exposed to the aqueous system [43]. Conversely, the CRL was capable to hydrolyse the test substrate in the o/w *ME IV* formulation, indicating that a *p*-xylene organic phase and Triton X-100 surfactant guarantee the lipase activity. A similar result was obtained with CAL which in *ME IV* maintains a satisfactory level of lipase activity.

We thus decided to perform the microemulsion biocleaning tests by using *ME IV* with both lipases (CRL and CAL). The same microemulsion was also tested without any enzyme.

The impact of the lipase on the structure of *ME IV* was investigated by measuring the radii of the droplet particles with the dynamic light scattering technique (DLS).

DLS measurements were performed using a Zetasizer-NanoS (Malvern) instrument operating with a 4 mW He-Ne laser (λ = 633 nm) and a fixed detector angle of 173° (non-invasive backscattering geometry NIBS™), with the cell holder maintained at 25 °C by a Peltier element. Data were collected after automatic optimisation of the instrumental parameters (attenuator, optics position and number of runs). The autocorrelation function (ACF) of the scattered light intensity was usually the average of 12-16 runs of 10 s each. The ACF of the scattered light intensity was converted into the ACF of the scattered electric field. The latter is the Laplace transform of the intensity weighted size distribution function that was retrieved using a standard regularised non-negative least squares analysis, operated by the software implemented by the manufacturer. The obtained intensity weighted size distribution function represents the fraction of the light intensity scattered by particles of different sizes.

The DLS data showed that the *ME IV* system without lipases is formed by w/o swollen micelles with an average hydrodynamic radius of 14.5±0.5 nm; the size distribution of these micelles is quite broad, with a polydispersity index (PdI) of 0.40±0.01. Addition of lipase to this formulation considerably reduced micelle size: the hydrodynamic radius with the enzyme is 9±1 nm with a very low PdI of 0.18±0.03. Interestingly, the type of lipase does not influence this marked decrease in micelle size; the addition of CRL or CAL actually gives the same micelle size.

This result might be due to the fact that lipase is an interfacial enzyme. The size of an o/w microemulsion micelle depends on the ratio between the amount of solubilized oil and the amount of surfactant at the interphase [44]. Lipase tends to be adsorbed at the water-oil interphase, where it evidently behaves as a surfactant, thus reducing droplet size.

2.3 Evaluation of the biocleaning tests

Stereomicroscope

For each zone subjected to the cleaning treatments, a stereomicroscope LEICA S8APO connected to a Nikon D5700 reflex digital camera took four photographs at different magnifications (1x, 2x, 4x and 8x).

Colour measurement and reflectance spectra

The chromatic properties and the reflectance spectra of the treated and untreated surfaces were measured using an X-Rite-i1-Pro spectrophotometer. Colour shifts were expressed as colourimetric values according to the CIE L*a*b* procedure using the three variables ΔL*ab, Δa* and Δb*. The magnitude of the global colour variation is given by [26]:

$$\Delta E^*_{ab} = \sqrt{(L^*_1 - L^*_2)^2 + (a^*_1 - a^*_2)^2 (b^*_1 - b^*_2)^2}$$

For each zone, the colourimetric data (L*, a*, b*) were an average value of three measurements on five spots. ΔE*ab values are an average of five ΔE*ab values calculated and the standard deviation was estimated on these values. The ΔE*ab were estimated by considering L^*_1, a^*_1 and b^*_1 relative to the untreated area and L^*_2, a^*_2 and b^*_2 of the treated layer. The higher the ΔE*ab, the higher the cleaning effectiveness

Five spots and three measurements were acquired also for the reflectance spectra used to evaluate fading and chromatic variations.

The area of the reflectance spectra (R) was calculated using the integration tool of Origin Lab 8.0 software. For this calculus the mathematical area (the algebraic sum of trapezoids) was selected. The difference between the areas of the reflectance spectra acquired on the treated and untreated zones (ΔR) was used to evaluate the cleaning effectiveness.

Image processing

The photos obtained with the stereomicroscope at 1x magnification were selected as being more representative of the treated area, and were used to calculate the percentage of white as a measure of the cleaned surface. The images were processed using Image J software to calculate the percentage of a selected colour, in this case white, which may be considered indicative of the cleaned areas.

Image processing involved the following procedure: briefly, a 24-bit image was calibrated and then the "thresholding colour" function was applied to equalize the coloured area to a single-level. For example, all green shades were levelled to only one shade. Then the image was converted into an 8-bit image (in greyscale) to apply segmentation by using the "thresholding greyscale" function to distinguish the object from the background. Once the objects were successfully segmented, they could be analysed using the “analyse particles” function to provide the percentage of white areas (%W).

3. Results and discussion

The biocleaning tests were carried out using the lipases in aqueous systems and in the *o/w* microemulsions. In particular, the mixture of bacterial lipases was used in aqueous system *AQ1*, as suggested by the vendor company, whereas CRL was tested in aqueous system *AQ2*. Both CRL and CAL were also tested in the selected o/w microemulsion system (*ME IV*) (Figure 1). All cleaning methods were applied to both aged and non-aged ink films on unglazed ceramic substrates using marker pens (test specimens).

Biocleaning effectiveness was evaluated by 1) the acquisition of reflectance spectra and the calculus of the difference between the spectral areas of the treated (cleaned) and untreated (uncleaned) zones (ΔR); 2) the determination of the colour differences (ΔE*ab) pre- and post-cleaning treatment; 3) image processing to determine the white percentage of the treated (cleaned) areas (%W).

The most relevant results are shown in Table 3. As a general consideration, the data show that the three evaluation methods may be considered quite comparable. In most cases, a high ΔE*ab value corresponds to high values of %W and ΔR. However, all methods show some limitations. Determination of the colour difference (ΔE*ab) provides an overall indication of colour change, which may not be strictly related to the amount of ink removed. Although the ΔE*ab value is frequently used in literature to measure the "degree of cleaning", it should be stressed that the use of this parameter should be limited to the comparison of different cleaning methods on the same substrate/artefact [26]. For this reason, the use of ΔR could help in correcting data interpretation. Indeed, positive values indicate a high reflectance percentage due to the surface of the white area as a result of ink removal. On the contrary, negative ΔR values may be explained by darkening of the area after the cleaning treatment.

The Image J processing that was proposed in this study as an additional evaluation method allowed us to obtain a percentage of white that might also be related to cleaning effectiveness. Unlike the colourimetric measurements this technique allows to estimate cleaning effectiveness by considering an area instead of point measurements. However, calculation of the percentage of white might be influenced by surface roughness, because shadows may disturb the threshold function.

Specific considerations about the effectiveness of the different biocleaning systems for the different inks may be proposed following detailed analysis of the ΔE*ab, ΔR and %W values, and considering the different types of ink (Table 3).

Samples	non-aged				artificially aged				naturally aged			
	%W	ΔE*ab	σ (ΔE*ab)	ΔR	%W	ΔE*ab	σ (ΔE*ab)	ΔR	%W	ΔE*ab	σ (ΔE*ab)	ΔR
P1												
AQ1 + Lip	60	28.0	± 3.2	83	50	24.1	± 1.8	66	35	19.7	1.7	54
AQ1	42	12.4	± 2.2	38	12	5.8	± 0.3	-6	24	5.1	1.1	2
ME IV+ CAL	42	19.8	± 1.3	65	32	25.7	± 1.2	79	14	6.7	1.4	17
ME IV	33	20.7	± 1.8	59	45	27.4	± 2.2	82	23	8.7	0.9	32
ME IV+ CRL	50	27.9	± 1.6	90	57	29.2	± 1.2	94	29	15.0	2.2	44
AQ2 + CRL	22	18.0	± 2.8	68	15	7.8	± 1.1	8	10	4.0	1.3	5
AQ2	16	11.7	± 1.2	18	6	7.3	± 0.9	14	10	10.1	0.7	25
P2												
AQ1 + Lip	21	28.3	± 2.9	46	48	20.17	± 2.3	8	49	8.4	± 1.6	1
AQ1	14	8.1	± 1.9	1	60	8.3	± 0.3	-24	38	4.0	± 1.6	18
ME IV+ CAL	51	27.8	± 0.6	47	49	11.1	± 0.6	-4	42	6.3	± 0.7	10
ME IV	28	22.4	± 2.3	47	41	12.3	± 2.1	-2	53	5.1	± 1.5	1
ME IV+ CRL	46	25.2	± 1.2	40	57	9.1	± 2.0	17	50	5.4	± 1.5	4
AQ2 + CRL	19	11.7	± 0.9	-6	29	13.2	± 2.1	-46	77	3.5	± 1.1	-8
AQ2	11	9.2	± 0.8	-2	30	12.3	± 2.3	-41	50	4.0	± 1.0	3
P3												
AQ1 + Lip	4	4.0	± 1.3	-2	23	5.4	± 0.7	19	77	5.4	± 1.1	18
AQ1	4	4.5	± 0.6	-3	32	7.2	± 0.8	62	28	2.3	± 0.9	7
ME IV+ CAL	88	36.2	± 2.3	101	37	9.7	± 1.6	79	37	6.6	± 1.1	30
ME IV	13	7.2	± 1.2	1	35	10.6	± 1.9	115	32	8.0	± 1.1	42
ME IV + CRL	7	4.3	± 1.2	20	55	18.8	± 2.0	65	72	22.6	± 1.2	75
AQ2 + CRL	5	5.2	± 0.7	2	34	7.3	± 0.8	-2	32	7.6	± 0.8	32
AQ2	10	4.3	± 1.2	-4	28	9.8	± 1.1	91	23	7.0	± 0.8	33
P4												
AQ1 + Lip	41	37.1	± 1.6	51	15	12.0	± 1.3	4	46	22.6	± 1.0	-16
AQ1	5	7.6	± 1.6	-3	19	15.2	± 1.1	-19	21	10.9	± 1.3	-37
ME IV+ CAL	69	35.5	± 0.7	50	22	17.0	± 2.0	14	30	14.3	± 2.6	-38
ME IV	29	28.2	± 1.0	44	29	24.0	± 1.1	-17	30	18.9	± 2.7	-40
ME IV + CRL	35	21.6	± 1.3	40	29	15.3	± 1.2	3	35	12.5	± 2.4	-30
AQ2 + CRL	30	21.2	± 1.6	33	22	19.6	± 1.2	4	28	19.5	± 1.2	-43
AQ2	18	10.6	± 1.2	6	21	18.3	± 1.0	-11	28	19.1	± 1.3	-39
P5												
AQ1 + Lip	9	12.4	±1.1	4	5	8.8	±0.2	2	2	1.3	±0.1	0
AQ1	8	5.8	±1.4	6	5	7.3	±0.4	15	3	1.8	±0.7	2
ME IV+ CAL	13	12.0	± 0.7	-1	1	4.2	±0.4	0.2	5	2.7	±0.0	-2
ME IV	5	6.7	±0.1	4	9	3.2	±0.3	8	2	2.2	±0.4	0
ME IV + CRL	4	6.4	±0.9	-3	6	3.8	±1.3	2	1	1.2	±0.4	-11
AQ2 + CRL	2	4.9	± 0.5	5	1	1.9	±0.4	-3	3	0.7	±0.5	-5

AQ2	2	8.5	± 1.1	8	5	2.9	±0.5	3	4	0.9	±0.5	-9
P6												
AQ1 + Lip	3	6.6	±0.3	-3	7	4.2	±0.2	18	7	7.4	±0.7	4
AQ1	5	6.8	±0.4	-11	4	3.4	±0.0	3	13	6.2	±0.8	15
ME IV+ CAL	4	4.2	±0.8	-11	7	3.4	±0.8	4	11	4.5	±0.2	4
ME IV	2	2.9	±0.1	-2	5	2.3	±0.3	6	3	2.9	±0.0	3
ME IV + CRL	4	4.9	±0.1	-8	4	5.3	±0.4	3	3	3.2	±0.4	3
AQ2 + CRL	0	3.9	±0.2	-14	6	3.8	±0.4	7	4	5.3	±0.5	7
AQ2	1	5.7	±0.3	-15	9	2.4	±0.3	9	1	7.5	±0.2	5

Table 3. %W, ΔE*ab and ΔR values calculated for the different cleaning methods applied to the aged and non-aged marker pen inks.

Uniposca green marker (P1). The cleaning tests performed on sample P1 gave encouraging results, especially on the non-aged samples. The *AQ1* formulation appeared very efficient when the enzyme was added, as shown by the net increase of values in Table 3. In particular, the microemulsions gave good results for the non-aged and artificially aged samples when *Candida rugosa* lipase was used (*ME IV* + CRL).

Results from the artificially aged samples are reported in Figure 2; satisfactory ink removal was achieved using *AQ1* with the enzyme (mix of lipase) and *ME IV* with and without enzymes. It can also be observed that the histograms for the different evaluation methods show the same trend, showing a good agreement with each other. The lower part of the figure reports the reflectance spectra recorded before and after cleaning treatments. It is evident that there was a net increase of values on specimens where cleaning was efficient.

Compared to the artificially aged samples, for the naturally aged ones the cleaning effectiveness decreased for all the tested systems, although the *AQ1* plus enzyme formulation maintained a good level of performance.

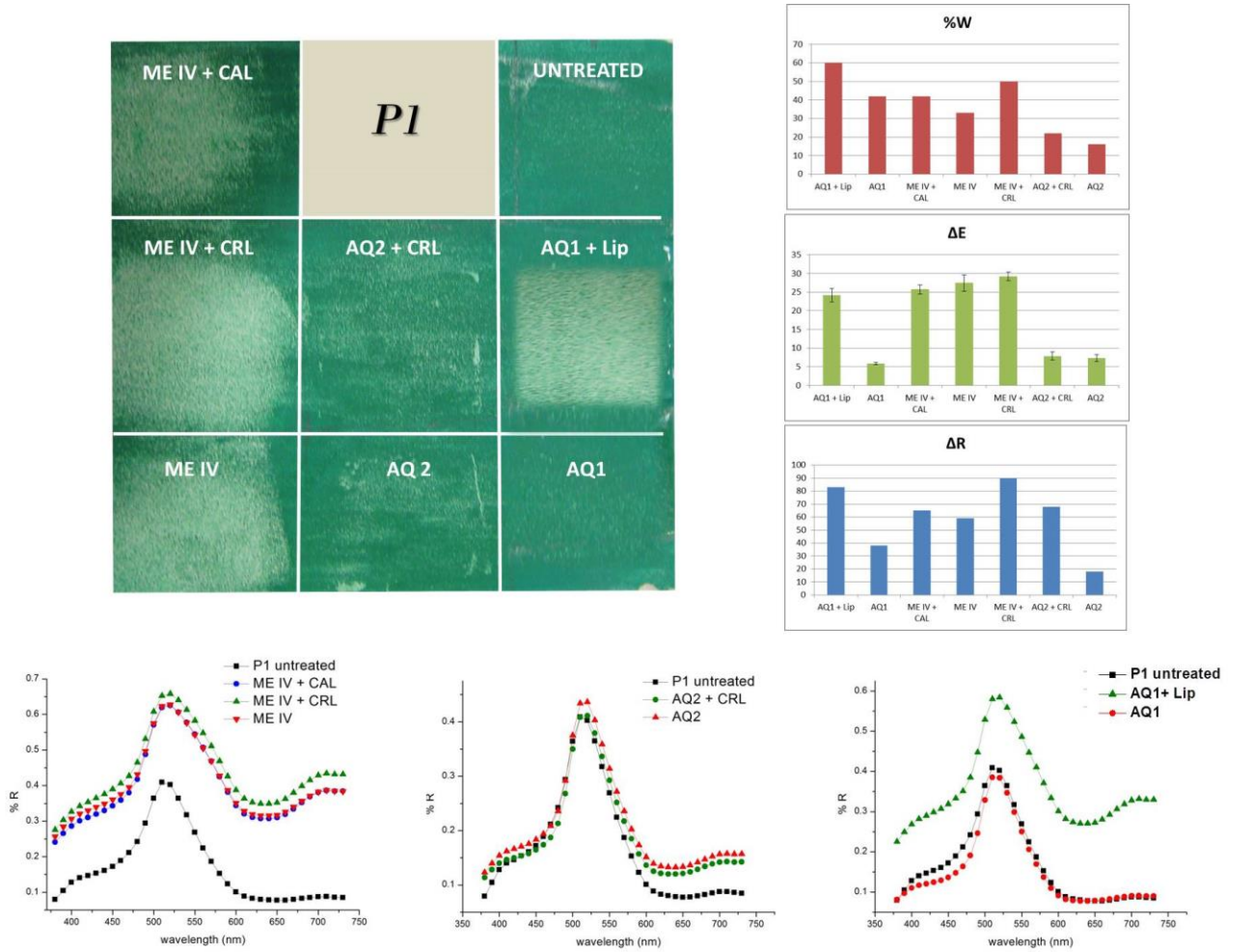

Figure 2. Cleaning tests performed on the artificially aged green Uniposca marker ink (P1). On the left: test specimens with the relevant cleaning system. On the right: histograms of the %W, ΔE*ab and ΔR calculated for each test. Reflectance spectra recorded before and after cleaning treatments with and without enzymes are reported in the lower part of the figure.

Red Uniposca marker (P2). Table 3 shows that the non-aged ink was already successfully removed with the use of bare microemulsions, and that there was a slight enhancement when a lipase was added; *AQ1* was effective only in combination with lipase, and *AQ2* was ineffective with and without the enzyme. The situation changed, however, when the tests involved aged samples. In this case, none of the methods seemed to work.

Results were negative for the aged samples: here it should be pointed out that examination of the reflectance spectra reported in Figure 3 showed that the photo-oxidative ageing already caused a marked loss of red paint. The red marker pen (P2) apparently contains the same type of binder as the

green one (P1) (see Table 1), for which positive cleaning results were obtained on both fresh and aged specimens. However, apart from the type of pigment, Py-GC-MS analysis [19,32] revealed a different relative abundance of the binder monomers: in the green marker ink MMA > 2EHA> nBA, whereas in the red sample 2EHA > MMA > nBA. The difference in sensitivity of acrylates and methacrylates to photo-oxidation could explain the cleaning results for the aged samples. Acrylate units are generally more reactive towards oxidation than methacrylates; moreover, cross-linking reactions prevail over chain scission for long ester side groups [45]. MMA is more abundant in the green marker ink; its photostability is well known and photo-induced oxidation requires severe accelerated ageing conditions [46]. P1 appeared unchanged after artificial ageing in our laboratories, and this facilitated its removal since the ester bonds of the acrylic portion were probably still intact; on the other hand, after natural ageing, the P1 ink layer looked more faded, probably due to the synergistic action of different factors, including relative humidity, temperature, pollution and solar irradiation.

The greater abundance of the less photo-stable 2EHA in the red sample may be responsible for major cross-linking, thus generating a polymer which is more difficult to remove and impeding lipase action.

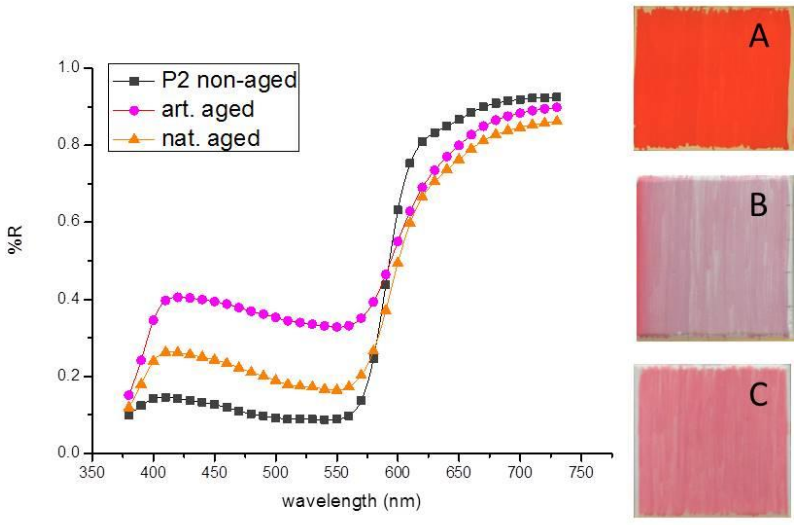

Figure 3. Test specimens with red Uniposca marker pen P2. On the right: before (A) and after artificial (B) and natural (C) ageing. On the left: reflectance spectra recorded on A (squares), B (circles), and C (triangles).

Blue Uniposca marker pen (P3). The data in Table 3 show that for the non-aged samples the addition of CAL in the microemulsion system *ME IV* successfully removes the ink layer, as shown in Figure 4. The CRL and CAL lipases used in this research are known to be non-specific, and their isoenzymes

actually share over 80% of sequence identity; however, they diverge in the lid sequences. In the active enzyme conformation, the open lid participates in substrate-binding and contributes to recognition of the substrate. This might explain the greater activity of CAL compared with CRL on the non-aged P3 sample. On the contrary, CAL activity seems reduced on the aged sample. Concerning the aqueous systems, both *AQ1* and *AQ2* methods, with and without the enzyme, proved ineffective on the aged samples.

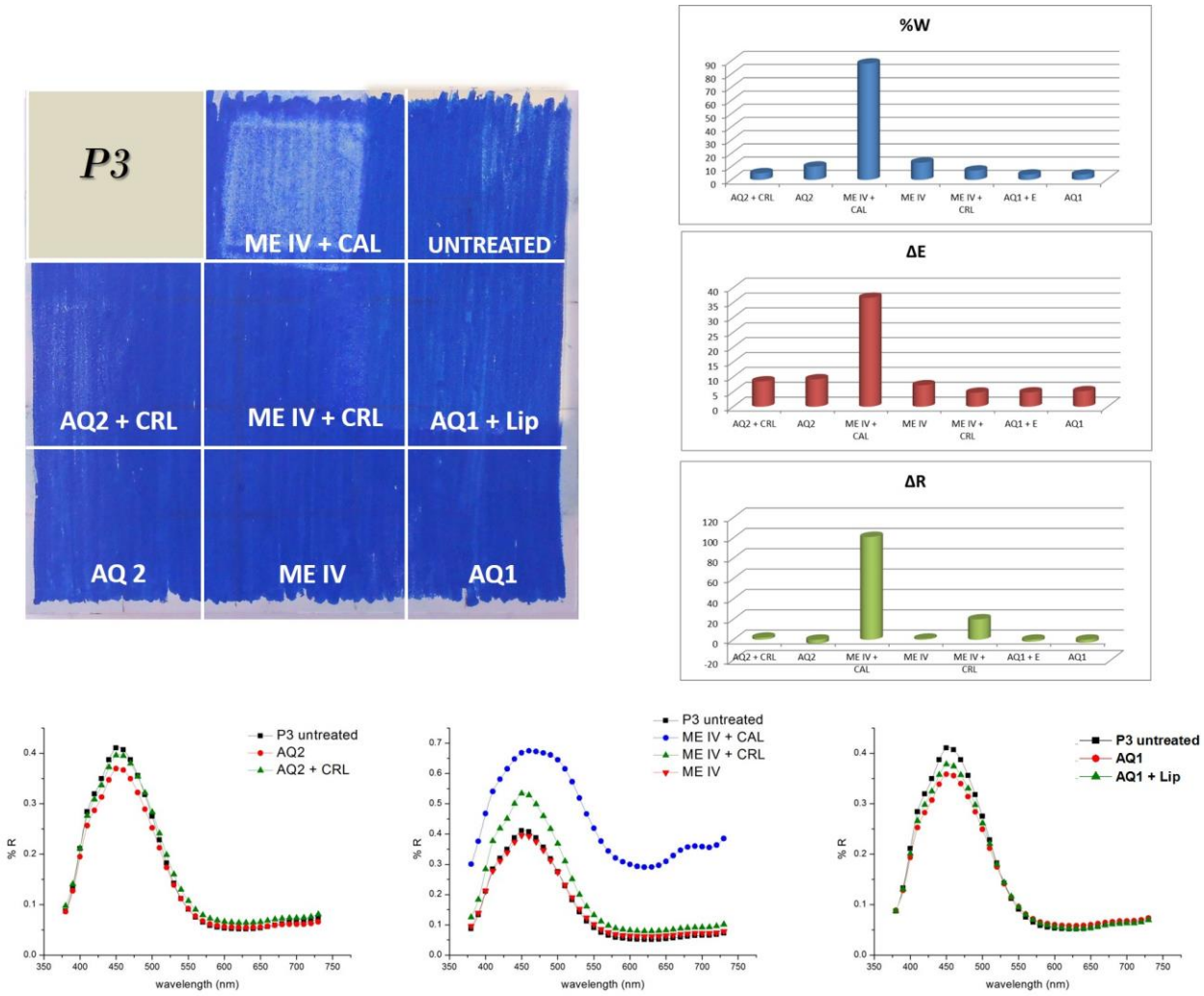

Figure 4. Cleaning tests on the non-aged blue Uniposca marker pen (P3). On the left: Test specimens with the relevant cleaning system. On the right: histograms of the %W, ΔE*ab and ΔR for each test. Reflectance spectra, recorded before and after cleaning treatments with and without enzymes, are reported in the lower part of the figure.

Red Tratto marker pen (P4). From analysis of the data in Table 3 and in Figure 5 regarding the non-aged samples, the aqueous systems with enzymes (*AQ1* + Lip and *AQ2* + CRL) appeared to be the most efficient, and microemulsion *ME IV* + CAL once again performed better than *ME IV* + CRL.

The cleaning effectiveness of *AQ1*+ Lip decreased only for the artificially aged samples, for which the microemulsions and *AQ2* + CRL systems gave good results.

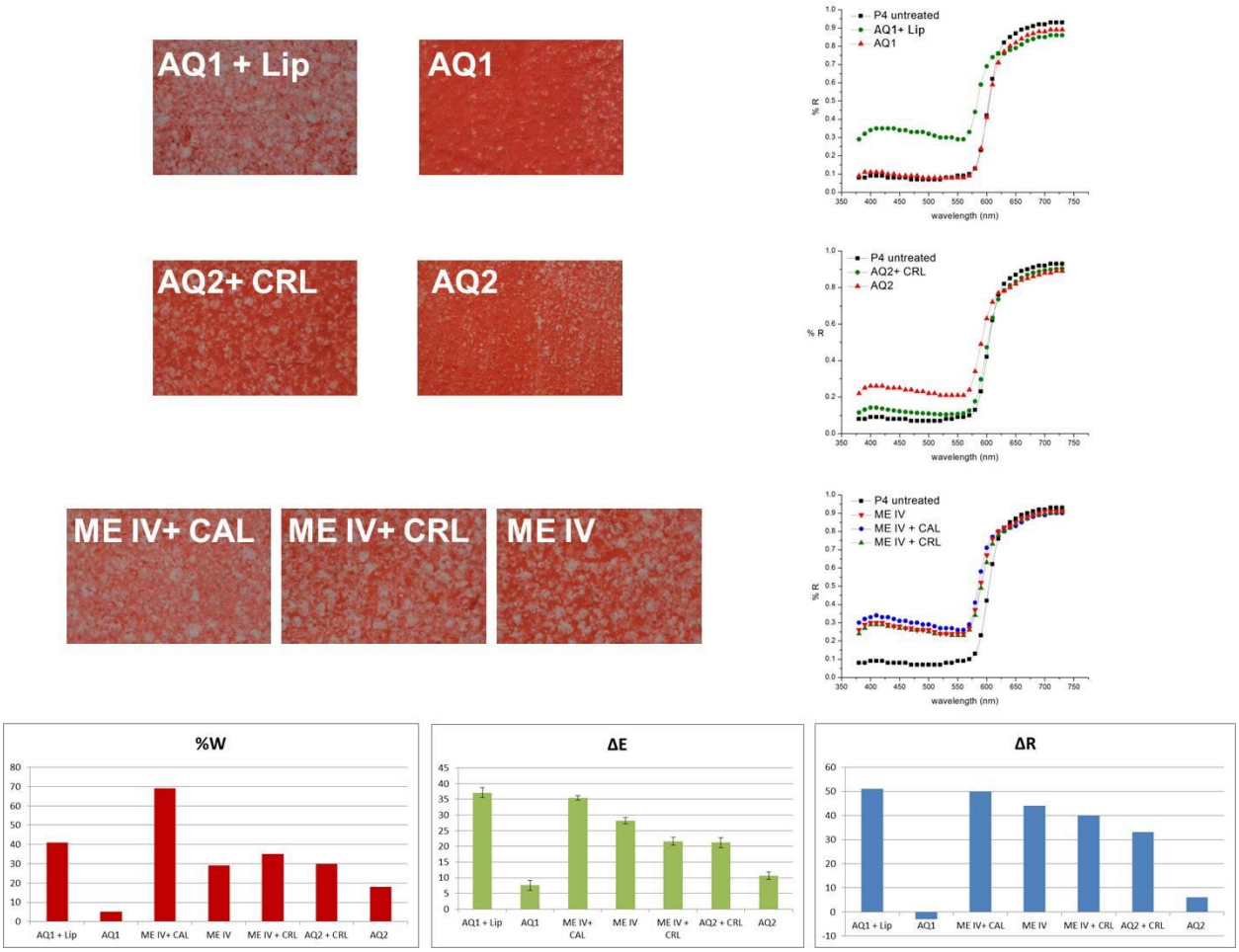

Figure 5. Cleaning tests on the non-aged red Tratto marker pen (P4). On the left: 2X magnification of the areas treated with different cleaning systems. On the right: reflectance spectra recorded before and after biocleaning. Histograms of the %W, ΔE*ab and ΔR for each test are in the lower part of the figure.

It is worthy to note from the results in Table 3 that none of the adopted cleaning methods was successful in removing the **green Tratto marker pen (P5)** or the **black Saratoga marker pen (P6).** In the case of the former, this may be due to the presence of a metallic component; X-ray Photoelectron Spectroscopy identified tin in the ink film (data not shown) and it is well known that this metal can inhibit the enzymatic action.

In the case of P6, the expectedly negative results of the biocleaning tests may be reasonably explained by the low amount of acrylics [19,32] in the binder, which is essentially composed of a copolymer of styrene and α-methyl styrene.

4. Conclusions

The study proposed a sustainable and green approach to the removal of marker pen inks. The method is based on the catalytic activity of the enzyme lipase. Some promising results were obtained in the removal of acrylic polymer-based inks with o/w microemulsions; in few cases these systems were effective even without the enzyme, but cleaning effectiveness was generally enhanced when a lipase was added.

Our findings can be summarized as follows: 1) All tested formulations were more effective on the non-aged samples, suggesting that biocleaning should be performed as soon as possible after blotting. 2) Among the cleaning formulations tested, there is no single formulation that is effective for all kinds of marker pen used, suggesting that simulation specimens should be correctly prepared and tested. 3) Acrylic binders containing a main monomer that is relatively stable to photo-oxidation, such as MMA, are more affordable substrates for lipases than the less photo-stable acrylic resins, possibly because the latter tend to lose the alkyl side chains or are subject to cross-linking which might inhibit the enzymatic attack. This suggests the necessity of a preliminary investigation on the chemical composition of marker pen inks in order to select the most appropriate biocleaning system. 4) The results clearly illustrate that only a multi-technique approach can correctly evaluate the effectiveness of different cleaning methods. To date, there is no standard methodology to evaluate the effectiveness or harmfulness of cleaning procedures, and this makes it difficult to compare results obtained in different conditions (laboratories, cleaning methods, substrates). An attempt to create an unequivocal evaluation method has been made only for laser cleaning methods (UNI 11187-2006), but should be extended to other cleaning techniques. 5) Finally, no cleaning method completely removed the ink from the unglazed ceramic substrates; however optimal results may be achieved by coupling the enzyme based cleaning step with a subsequent very mild mechanical/chemical approach, thus preserving the integrity of the substrate.

In the future, an evaluation of the lipase activity residues with FT-IR spectroscopy will be carried out as well as an estimation of the penetration of the cleaning formulation in the substrate.

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